Beyond the Bubbles

A Study of Fungal Diversity and Gushing in Beer Production

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Bortom bubblorna - Svampdiversitet och överskumning vid ölproduktion

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Abstract

Gushing, violent and spontaneous over-foaming of beer, is a well-known quality reducing phenomenon encountered in the brewing industry. The present study investigated the impact of fungal contamination in barley grains, specifically focusing on gushing and the occurrence of pink kernels. A total of 101 seed samples of the malting barley cultivar Planet, harvested in 2020, were sourced from three seed handling sites in the southern and central parts of Sweden - Malmö, Lidköping, and Köping. The fungal communities within these samples were characterized using amplicon sequencing. Furthermore, machine learning techniques were employed to estimate the relative importance of these communities in inducing gushing and in forming pink kernels. Our findings reveal a diverse assembly of fungal species, including yeasts and filamentous fungi, across the analysed samples. The most common fungal genera were *Fusarium*, *Cladosporium*, *Alternaria*, *Vishniacozyma*, and *Sporobolomyces*. Particularly, a *Fusarium* species complex was identified as being associated with both the formation of pink kernels and the induction of gushing, emphasizing its implications to malting barley production. Additionally, this study also marks the first report of the fungal genera *Vishniacozyma*, and *Itersonilia*, in the context of pink kernels and malting barley research, highlighting their unexplored role in this context. The results point to the necessity for close monitoring of diseases in malting barley and the implementation of effective management strategies, such as improved crop rotations, in order to ensure that the quality and safety of malting barley meets industry requirements.

Keywords: Fusarium Head Blight, Fungal communities, Metabarcoding, Amplicon sequencing Malting barley, Gushing, Pink kernels, Random forests

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Abbreviations

FHB	Fusarium head blight
ITS	Internal transcribed spacer
SH	Species hypothesis
RF	Random forest
RSS	Residual sum square
IV	Independent variable

1. Introduction

1.1 Background and aims

Gushing, the violent and spontaneous over-foaming of beer, is a well-known quality reducing phenomenon encountered in the brewing industry. The phenomenon is divided into primary gushing, induced by fungal activity, and secondary gushing, caused by the presence of colloidal or solid particles or excess amounts of carbon dioxide (Lusk 2016). While secondary gushing can be avoided by good manufacturing practices, primary gushing is less well understood, and causes challenges in the beer brewing and seed handling industry. Grain contamination with fungi within the genus *Fusarium* has been identified to be particularly important in problems with primary gushing. Therefore, presence of pink kernels, a symptom of *Fusarium* infection, is used as a quality indicator in malting barley used for beer production. However, the role of non-*Fusarium* fungal species in gushing and the presence of pink kernels remains underexplored. Filling this knowledge gap has the potential to improve environmental sustainability in malting barley production by reducing the number of unnecessary applications of fungicides. Social sustainability can be addressed by minimizing the erroneous rejection of seed lots, and improvements in economic sustainability arises from increased profitability for the farmer by disseminating the relationship between gushing, pink kernels, and fungal communities.

1.1.1 Aims

This study aims to contribute to gushing and malting barley-related research by examining the influence of fungal contamination of barley grains on gushing and the presence of pink kernels. To explore this, the present study addressed the following research questions:

1. Which fungal species are represented in the fungal communities in barley grains?
2. Which fungal species are associated with the gushing-propensity of the collected barley samples?

3. Which fungal species contribute to the presence of pink kernels in barley grains?

The primary research objective of the present study was to identify fungal species that are relevant in the context of fungal contamination of malting barley leading to the presence of pink kernels and/or the induction of gushing. To achieve these objectives, fungal communities on Swedish malting barley grains from the growth season of 2020 were characterized using amplicon sequencing. The data was analyzed using supervised and unsupervised machine learning techniques, including the construction of classification-based random forests and dimensional reduction techniques.

The significance of this study's results lies in its potential implications for the beer production industry. By identifying the specific fungal species associated with gushing and the presence of pink kernels, the findings provide information which can be used to develop targeted strategies for improving the quality assessments of barley grains by offering insights into how fungal community composition differs in samples that induce various amounts of gushing. This could in turn lead to a more sustainable production process that has the potential to minimize the economic losses experienced by farmers due to fungal contaminated grain.

1.2 Brewing business: Europe's beer industry

European beer markets | The 19th-century European beer market was characterized by dominant local and national monopolies. Recent shifts in the European beer market reveal that there is an increased abundance of transnational corporations, such as the Carlsberg Group, and Heineken. The presence of large-scale transnational corporations indicates that the beer market has reached a point of maturity wherein acquisition strategies are the main way of increasing market capital (Hána et al. 2020). Furthermore, the European beer market is experiencing annual growth, and the total amount of beer produced in Europe is increasing. Eurostat – Europe's statistical office – noted an overall 20% and 3% increase in the production of alcoholic and non-alcoholic beer, respectively, between the years 2020 and 2021 (Eurostat 2022). Beer production decreased in most European countries during 2020, most likely due to the advent of the COVID-19 pandemic in Europe and the subsequent disruption of regional and transnational trade networks (Figure 1).

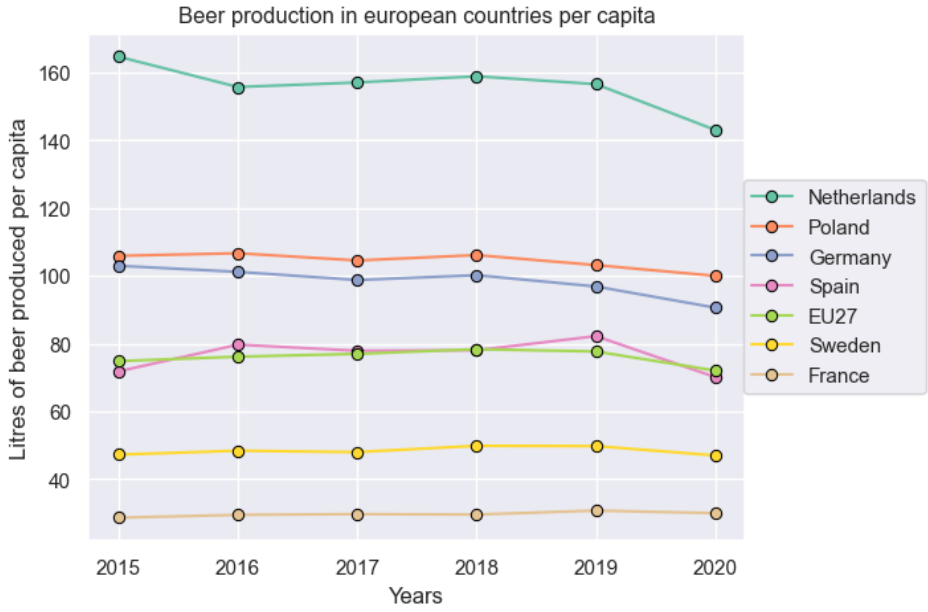

Figure 1. The production of alcoholic beer (>5% alcoholic content by volume) in European countries per capita over a six-year time-period. Source: Eurostat DS-056120 & Eurostat TPS00001.

Prospects of Swedish beer market | In Sweden, recent brewery-related market reports have revealed an increase in the formation of new breweries, indicating that the Swedish beer market is less mature than that of the beer market of the European inland. Indeed, between 2010 and 2021 the number of breweries in Sweden increased more than 50-fold, totalling 534 breweries. Most of these new breweries

are, however, either micro- or nano-breweries producing artisanal beer, meaning that the overall production capacity of such breweries is considerably lower than that of industrial breweries (SPAA 2022).

Malt, a precursor to beer | Malt, a precursor in malt beer production, is commonly produced from the kernels of barley. The process of making malt itself follows three generalized steps- steeping, germination, and kilning, with quality control taking place before and after the malting process to verify the product for downstream applications. The maltster is usually separated from the farmer and the brewer, and acts as a middleman malting the grains which are then sold to separate breweries. Typically, industry-standardized two-rowed malting barley cultivars indexed with malting grades must be sown, cultivated, and harvested. The harvested grain needs to meet certain quality standards before undergoing malting. Examples of such quality standards include varietal purity, moisture content, and protein content. Malting barley is generally recognized as a premium ware, primarily due to the precautions farmers need to take to ensure their product meets the quality standards. Adverse climactic field conditions such as drought or waterlogging, or the presence of diseases during the vegetation period can result in the deterioration of barley grain quality, potentially triggering detrimental biochemical reactions during the malting process, such as the accumulation of mycotoxins or gushing-related factors respectively. This can in turn render the barley unsuitable for human consumption, relegating unsatisfactory batches to be used as livestock feed. Livestock batches of malting barley are not sold as a premium ware, and as such holds a lower market value than that of malting barley (Briggs 1998; Geißinger et al. 2022).

1.3 Multidimensional Sustainability

Barley production in Europe | Barley is ranked as one of the five most important cereals for global food production and trade, with a stable annual production that has not changed significantly in the past 15 years. The European union is the main producer of barley from a global perspective totalling 53 million tonnes 2022 (Lukinac & Jukić 2022). Recent socio-political events such as Russia's invasion of Ukraine is expected to affect the global supply of various agricultural commodities such as sunflower oil, wheat, and barley. The disruption caused by the invasion is expected to reduce Ukraine's agricultural production of spring sown crops in 2023 by 20%, and the reduction in export of agricultural goods from Ukraine undermines food security in countries which are dependent upon Ukraine's exports (Gay et al. 2022).

Socio-political challenges in agriculture | The on-going energy crisis in Europe is also fuelled by the previously mentioned socio-political events. The prices of energy commodities such as gas and electricity are steadily increasing, which puts

agricultural sustainability in a vulnerable position – possibly jeopardizing European sustainability incentives related to agriculture, such as the farm to fork strategy which aims to make Europe a climate-neutral continent (Fiore et al. 2022). The upward trend in gas and electricity prices has a direct impact on various industries and organizations. Modern industrialized agriculture is dependent upon energy-intensive equipment such as tractors and combine harvesters, as well as off-farm supply chains, and relies heavily on the usage of gas and fossil fuels as an energy source, thus making food production susceptible to fluctuations in energy prices. The destabilizing effects on the European energy market therefore risks putting food-sovereignty and environmental sustainability at odds, resulting in a trade-off scenario (Borowski 2022). In such a scenario ecological farmland or natural forest may be repurposed to large-scale agricultural production, while the overall productivity is predisposed to decrease at the same time, if the cost of applying agricultural inputs due to an increase in gas prices surpasses that of the potential economic gains at harvest, leading to a cycle of changes in land-use and increasing greenhouse gas emissions (Agnoletti & Santoro 2022; Blöschl 2022). A reduction in the diversity of cultivated crops within an agricultural production setting consequently diminishes the dilution effect. The dilution effect is an ecological concept that denotes the resilience of an ecosystem against potential pathogenic organisms (Pretty 2008; Khalil et al. 2016).

Environmental sustainability in Europe | The homogenization of production settings at a landscape scale includes reduction of complexity in crop rotations and deforestation. Homogenization caused by policies relating to the energy crisis may therefore elevate the incidence of plant-pathogens and disease in fields as per the dilution effect. Superimposed elements such as plant pathogens affect productivity at field-level. Plant diseases reduce wheat yields by 10-28% (Savary et al. 2019), and FHB which is caused by a conglomerate of *Fusarium* species similarly reduces quantitative yields by up to 26% in barley (Nganje et al. 2004). The need for greater environmental, social, and economic sustainability is therefore apparent in agricultural production systems in Europe especially in the face of climate change, which is expected to add additional layers of stressors to food production systems. Indeed, the 2022 climate change report from the intergovernmental panel on climate change (IPCC) elucidates the impacts of such stressors. Amongst these, a three-fold increase in droughts and heatwaves have been observed in Europe (Brás et al. 2021), and the pole-ward migration of important crop pests and pathogens (Bebber et al. 2013). The report then calls for increased utilization of ecosystem-based solutions such as agroforestry, which aids in the exploitation of different niches in agricultural production settings, as well as agroecological principles, both of which have the potential to increase food system resilience as well as productivity in the face of climate change (Kerr Bezner et al. 2022).

1.4 Quality parameters in beer production

Gushing in beer production | Gushing, a quality reducing phenomenon occurring during the brewing process is characterized by abrupt and excessive production of foam upon opening of storage containers. This undesired phenomenon results in substantial economic losses. Primary beer gushing is induced by the occurrence of filamentous fungi and dimorphic yeasts during the malting process, unlike secondary gushing which depends upon faults occurring during the brewing process, such as the addition of excess carbon dioxide (Virkajärvi et al. 2017a). Indeed, the prevalence of primary gushing during downstream production stages is closely associated with the presence of FHB infected grains used in the malting and brewing phases of beer production (Mastanjević et al. 2018). The mechanism behind primary gushing involves the presence of micro-bubbles within beer containers, with nucleation centres primarily attributed to the presence of fungal hydrophobins during the brewing process (Wolf-Hall 2007).

Hydrophobins as gushing-inducing proteins | Hydrophobins are a group of small, 7 – 15 kDa, globular proteins which are employed by fungi for aerial growth and hyphal adherence to solid surfaces. Hydrophobins are separated into two classes (class I and class II) depending on their solubility in acids (Linder et al. 2005; Shokribousjein et al. 2011). Exuded hydrophobins reduce water surface tension that results in the formation of an amphipathic interface. As the fungal hyphal network expands, newly exuded hydrophobins are intercalated into the amphipathic bilayer (Shokribousjein et al. 2011). In short, plant-pathogenic fungi utilize hydrophobins for attachment, and to stick to their hosts, which facilitates the early stages of the infection cycle (Paananen et al. 2003). In beer bottles hydrophobin concentrations in the range of 1 mg/L is sufficient to induce gushing, but this threshold value varies as per the type of hydrophobin (Sarlin et al. 2005b). Hydrophobins induce gushing by stabilizing microbubbles found in beer, and the change of internal pressure when opening a beer bottle causes a cascade effect of stabilized micro-bubble explosions, bubble re-nucleation, bubble growth, and finally bubble explosion – with these four steps occurring simultaneously (Shokribousjein et al. 2011).

Ascomycetes and hydrophobins | Class II hydrophobins is the only class which has been shown to induce gushing in beer and are primary metabolites of ascomycetes (Linder et al. 2005). Currently, gushing is primarily attributed to class II hydrophobin prolific species within the genus *Fusarium* (Linder et al. 2005; Minenko et al. 2014). Non-*Fusarium* fungal genera such as *Aspergillus* and *Penicillium* have also been reported to produce hydrophobins that induce gushing in beer (Virkajärvi et al. 2017a).

ns-LTPs as part of gushing | Other gushing inducing factors such as plant-derived non-specific lipid transfer proteins (ns-LTPs) are small ubiquitous proteins involved in the transfer of proteins across lipid membranes. Their functions in

plants include cuticle synthesis and loosening of the cell wall (Iqbal et al. 2023). As pathogenesis related proteins, ns-LTPs also have a functional role in host defence against invading phytopathogens by inducing the efflux of intercellular ions in the invading pathogen (Selitrennikoff 2001). The protein ns-LTP1 is abundant in the endosperm of barley grains, when present in normal amounts ns-LTP1 aids in the formation of beer foam. However, both abiotic and biotic stressors can induce the accumulation of ns-LTP1 in the endosperm, leading to an overproduction of foam, and induction of gushing when beer containers are depressurised (Hippeli & Elstner 2002).

Detecting gushing | Methods used for the detection and prediction of gushing inducing factors such as hydrophobins during beer production includes the doubly Modified Carlsberg Test (M2CT) and other similar varieties of it. M2CT emulates brewing conditions and measures the total amount of over-foaming from bottles measured in grams (Deckers et al. 2012). Dynamic light scattering (DLS) detects particle sizes in solution and therefore allows for the identification of micro-bubbles stabilized by hydrophobins – this method also allows breweries and researchers to discriminate between primary and secondary gushing due to the differential radius of the two types of micro-bubbles (Deckers et al. 2012). Finally, competitive enzyme-linked immunosorbent assays (ELISA) can be used for the detection of hydrophobins in malt extracts via the use of antibodies. However, ELISA fails to detect hydrophobin concentrations of less than 100 µg/mL (Sarlin et al. 2005b). As for the development of new methodologies, Stilman et al. (2022) developed a tabletop sensor that can detect hydrophobins at concentrations no lower than 30 ng/mL in beer, this method relies on impendence spectroscopy and the use of molecularly imprinted polymers.

Pink kernels as an indicator | The previously mentioned methods are only able to be used for quality verification once malting of the barley has taken place. Another indicator commonly used in industry to predict gushing is the presence of pink kernels in seed samples taken at farm level, before arrival at the maltsters. Due to the correlation between pink kernels and kernels infected with *Fusarium* fungi, this quality-verification step can be taken before the malting process which discards seed-batches with poor quality or high FHB incidence (Schwarz et al. 1996). The instrument CGrain Value™ is an example of pre-malting analysis of seed batches for the presence of pink kernels. CGrain Value™, developed by CGrain AB, automatically analyses seed batches for the abundance of seeds from agricultural weeds, pink kernels, and husk damage. Each seed is evaluated independently, and a top-down photo is taken of a kernel as it is moved through a chamber with optical mirrors allowing for a view of 90% of the surface of a given kernel of barley – the analysis is done at a rate of 8-12 kernels per second (Cgrain 2016).

FHB and mycotoxins in beer and malt | From a global perspective, FHB is caused by a complex of up to 20 different *Fusarium* species (Karlsson et al. 2021),

and infections are generally favoured by warm and humid climactic conditions (Rossi et al. 2001). Among the 20 causative agents of FHB, the four most common species causing FHB in Europe are *F. culmorum F. avenaceum, F. graminearum,* and *F. poae* (Karlsson et al. 2021). In addition to the yield losses caused by FHB, which vary between 10 - 30% in small grains, accumulation of mycotoxins in grain during both pre-, and post-harvest storage (Table 1) poses a risk to human and animal welfare (Bottalico 1998). The presence of *Fusarium* mycotoxins has been documented in beer from both artisanal and industrial breweries alike, where deoxynivalenol and zearalenone are among the more common mycotoxins found in alcoholic beer (Schabo et al. 2021). A typical indicator of FHB infections is the presence of pink kernels in seed lots. Pink kernels exhibit their pinkish discoloration due to the presence of polyketide pigments, which are secondary metabolites produced by many *Fusarium* species during their metabolic processes (Thomas & Tirumale 2022). One study found *F. avenaceum* to have a particularly high correlation with the incidence of pink kernels in seed lots from Slovakia (Hudec 2007).

Table 1. Mycotoxins commonly associated with FHB, their effects on cellular functions, and food content limitations for mycotoxins. Mycotoxins frequently found in beer has been marked with an asterisk (*) (Schabo et al. 2021). Cellular mechanisms adapted from Bertero et al. 2018, and food thresholds from Commission Regulation (EC) No 1881/2006 of 19 December 2006 setting maximum levels for certain contaminants in foodstuffs.

Mycotoxin	Cellular mechanisms in animals	Threshold in foodstuffs
Zearalenone (ZEA)*	Endocrine disruptor causing ovarian atrophy, and alterations to the reproductive tract.	100 mg/kg
Beauvericin (BEA)	Induction of apoptosis via calcium-dependent endonuclease, and endocrine disruption of hormone receptor transcription.	-
Deoxynivalenol (DON)*	Activation of mitogen activated protein kinase signalling pathways, leading to apoptosis and cellular damage. Ribotoxic stress, and inhibition of protein synthesis.	1 250 mg/kg
Fumosin-B_1 (FB_1)	Inhibition of sphingosine, leading to inhibition of cell regulation in blood and urine. Known carcinogen with species-, and gender-specific effects.	800 mg/kg
T-2 toxin (T2)*	Inhibition of RNA and DNA synthesis, induction of apoptosis and lipid peroxidation leading to immune system impairment.	200 mg/kg

1.5 Fungal community composition in barley

Fungal associations with barley kernels | The phyllosphere, or aerial and above-ground parts of plants, including flowers, fruits, and leaves are colonized by a conglomerate of prokaryotic, and eukaryotic microorganisms (Bashir et al. 2022). Revealing the microbial diversity in the phyllosphere can be accomplished through molecular analyses, such as metabarcoding via PCR amplification and sequencing of the internal transcribed spacer region (ITS) of fungi, or shotgun genomic approaches. The former provides a more focused view of a kingdom's phylogeny, while the latter offers broader insights into the genetic diversity within microbial communities (Quince et al. 2017; Nayfach et al. 2021). Nielsen et al. (2011) documented a negative correlation between the two *Microdochium* species *M. majus* and *M. nivale* and *Fusarium* in Danish grains (Nielsen et al. 2011). Similarly, a study by Rojas et al (2020) documented a reduction in diversity of fungal communities on wheat kernels when *Fusarium* species dominated. In the same study, the authors identified several fungal endophytes which have a negative correlation with including the fungal genera *Itersonilia*, and *Cladosporium*. The same study also documented a temporal variation of fungal clades over the growing season, where *Basidiomycota* were found to dominate the fungal communities early in the growing season, but a shift towards communities with a greater abundance of *Ascomycota* was observed during wheat kernel development and maturation (Rojas et al. 2020). The microbiome is indeed a complex and highly dynamic environment, with fungal communities being tissue and genotype specific while aboveground communities are more dynamic than that of fungal communities in belowground plant tissues such as roots (Malacrinò et al. 2023; Sapkota et al. 2023).

2. Materials and methods

The data analysis pertaining to this study builds upon data obtained from plant material collected in 2020. Ergosterol, DNA extraction, and amplicon sequencing was carried out during 2021-2022.

2.1 Methodological theory

2.1.1 Decision trees

The construction of decision trees follows the theory supplied in James et al. (2013). As a supervised machine learning algorithm, decision trees can be used for the prediction of either categorical or continuous dependent variables and are therefore known as either classification or regression trees. An individual decision tree is built upon the idea of data stratification, wherein the predictor space, or P possible values for an independent variable (IV): $X_1 \rightarrow X_P$, is divided into J number of distinct and non-overlapping regions: $R_1 \rightarrow R_J$. For each observation which is contingent to region R_j is assigned the same prediction, namely the mean response values for the training observations in R_j. For regression trees the stratification of data into separate regions is optimized to reduce the mean squared error (MSE) of the model. A residual is the difference between the actual value (y_i) and the predicted value within region R_j ($\hat{y}_{R_j}$), and the MSE is the average of the sum of all residuals squared (Equation 1); other split criterions are also common, residual sums squared, Friedman mean squared error, absolute error, and Poisson deviance.

Equation 1. Mean squared error (MSE), which is a metric used to evaluate the performance of a regression decision tree by quantifying the discrepancy between the actual target values (y_i) and the predicted values within each region R_j ($\hat{y}_{R_j}$)

$$MSE = \sum_{j=1}^{J} \frac{1}{n} \sum_{i \in R_j} (y_i - \hat{y}_{R_j})^2$$

Recursive binary splitting is used for the partitioning of the IV space, meaning that IV region splits are top-down and occur successively. All data is therefore pooled at the top of a decision tree. When conducting a split (Equation 2), the model

does not take future splits into account, and is therefore based upon the split which yields the lowest MSE at that point in the data partitioning process (Equation 3).

Equation 2. Two regions are defined as $R_1(j,s)$ and $R_2(j,s)$, the creation of which result from partitioning the predictor space based on a specific feature (j) and a split point (s). $R_1(j,s)$ comprises all the data points for which the value of the selected IV (X_j) is less than the value of s, whereas $R_2(j,s)$ encompasses all the data points for which the value of the selected IV is greater than or equal s.

$$R_1(j,s) = \{X | X_j < s\} \; and \; R_2(j,s) = \{X | X_j \geq s\}$$

Equation 3. The equation delineates the criterion for determining the optimal feature (j) and split point (s). This optimization process seeks to minimize the sum of squared residuals within the two resulting regions, R1(j, s) and R2(j, s), formed by partitioning the data based on the chosen feature (j) and its corresponding threshold (s). In the equation, y_i represents the actual target value for each data point, while $\hat{y}_{R_1}$ and $\hat{y}_{R_2}$ denote the respective mean predicted values in the regions R_1 and R_2.

$$\sum_{i:x_i \in R_1(j,s)} (y_i - \hat{y}_{R_1})^2 + \sum_{i:x_i \in R_2(j,s)} (y_i - \hat{y}_{R_2})^2$$

In a similar fashion to regression trees, classification trees rely on recursive binary splitting, but since the response is qualitative, the use of MSE is no longer possible. Instead, the minimization of the Gini index, or other classification-based error metrics can be used to evaluate the best the split for a given IV (Equation 4). A lower Gini index indicates a higher degree of purity within a given node of the decision tree, facilitating the identification of optimal IV splits during the construction of a classification tree.

Equation 4. The Gini index (G) is calculated by summing the product of the estimated class probability ($\hat{p}_{mk}$) in the m^{th} region that are from the k^{th} class, and its complement ($1 - \hat{p}_{mk}$) for each class k, where K represents the total number of classes.

$$G = \sum_{k=1}^{K} \hat{p}_{mk}(1 - \hat{p}_{mk})$$

2.1.2 Random forests

Random forests are aggregated prediction models using decision trees as building blocks to construct more robust models with lower variance, while simultaneously reducing the risk of overfitting. The result is a RF with a higher degree of reproducibility, partly due to employing bootstrap aggregation on the set of training data. Bootstrap aggregation is the process by which multiple new data sets are generated by repeatedly sampling data from the main training data set, finally the reduction of variance comes from averaging the observations in the bootstrap, which in turn yields a predictive model with reduced variance (Equation 5). In this manner hundreds of decision trees can be combined for more robust results.

Equation 5. A bootstrap aggregated ensemble model consisting of B number of base models for the input x ($\hat{f}_{bag}(x)$) is constructed by repeated sampling of the b[th] base model with input x ($\hat{f}^{*b}(x)$). The result is then averaged to reduce variance of the aggregated ensemble model.

$$\hat{f}_{bag}(x) = \frac{1}{B}\sum_{b=1}^{B} \hat{f}^{*b}(x)$$

Random forests utilize bootstrap aggregation together with a methodology built on randomness which decorrelates the individual trees that constitutes the random forest – hence their denotation as *random* forests. When a split is considered while constructing one of the base decision trees, a random sample of m IVs are chosen from the full set of p IVs as candidates for the split. This allows for the individual trees to be decorrelated, since on average strong IVs are not represented in m as long as $m \neq p$. Typically, $m \approx \sqrt{p}$ since it generally produces the lowest RSS or Gini index. The result from a random forest model is the relative importance of each IV for predicting the dependent variable, however the method varies between regression and classification problems. For regression random forests the total decrease of MSE (or other error measurement) for a given IV, averaged over all *B* base decision tree models are represented as that IVs relative feature importance. The methodology is similar for that of classification random forests, the decrease of the Gini index for a given IV is totalled and then finally averaged over all *B* decision tree models.

2.2 Sample collection

A total of 101 seed samples of malting barley cultivar Planet, harvested in 2020, were collected from three seed handling sites in the southern and central parts Sweden - Malmö, Lidköping, and Köping. An initial analysis testing for the presence of pink kernels in each of the samples was performed using a Cgrain Value™ image analysis instrument. Based on this, 70 rejected (≥0.1% pink kernels) and 31 accepted seed lots were identified. Parallel samples to those initially analysed were collected, the weight of which ranged from 417 to-1130 g.

The samples were stored in plastic bags for 6-8 months before further analyses. To exclude samples with risk for fungal growth during storage, only samples with a water-content of less than 15% were selected. The samples were cleaned using a Pfeuffer SLN4 sample cleaner, with aspiration for 60 seconds to remove dust, and sieving to remove weed and straw material. Seed sizes between 220 mm and 450 mm were categorized as potential barley seeds and were kept for further analyses. Aspiration and sieving were done by the Department of Soil and Environment, Swedish University of Agricultural Sciences (Uppsala, Sweden).

2.3 Ergosterol content

To estimate the ergosterol content of the samples, a near-infrared transmittance measurement assay was conducted using an Infratec™ NOVA 400 – 1100 nm monochromator using a calibration developed by FOSS (Hillerød, Denmark). Data was recorded using a silicon sensor with an optimal bandwidth of 7 nm. The ergosterol measurements were carried out at the Department of Soil and Environment, Swedish University of Agricultural Sciences (Uppsala, Sweden).

2.4 Image analysis

Seed samples were divided into sub-samples using a seed sample divider, into one sub-sample of 160 – 161 g (sub-samples A), and one of 50-51 g (sub-samples B). The content of pink-kernels in each sub-sample was determined using a Cgrain Value™ image analysis instrument. The sub-sampling procedure was repeated until the difference in pink kernel content between sub-samples A and B was less than 0.2%.

2.5 Modified Carlsberg test

Gushing potential of seed samples was analysed using Modified Carlsberg tests performed by the Research Center Weihenstephen for Brewing and Food Quality (BLQ) at the Technical University of Münich, Germany. Sub-samples A were coarsely ground and homogenized. 100 g of the coarse grist was blended for 60 seconds with 400 millilitres of distilled water in a laboratory mixer (15 000 rpm) at room temperature. The mixture was centrifuged (RCF of 4 500 g, 10 min) and 300 millilitres of the supernatant was reduced through boiling to a volume of 200 millilitres. Aliquots were measured and filtered from the supernatant and allowed to cool down to room temperature (20°C). 5 millilitres of sodium aside stock solution was added to each aliquot, which was then diluted with sterile water to a total volume of 200 millilitres. The 50 mL aliquot was added to each three bottles of standardized table water (0.33 L Bonaqa®) and the bottle was shaken horizontally for 72 hours at 20 °C. After the process of shaking and opening the bottle, the weight of liquid flowing out from the bottle was determined and taken as a measure of the gushing potential for the sample. The mean amount from three replicate bottles was used as the final value for gushing.

2.6 DNA extraction and amplicon sequencing

2.6.1 DNA extraction

Sub-samples B were finely ground and homogenized using a DeLonghi KG40 coffee grinder. For each sample two 0.1 g aliquots of ground barley grain were used for DNA extraction using the NucleoSpin® Plant II (Macherey-Nagel, Germany) and following the manufacturer's instructions with the following modifications: After adding the cell lysis Buffer PL1, samples were homogenized using a Precellys Evolution (2x5,500 RPM for 35 seconds) (Bertin Technologies) and vortexed. Subsequently, 10 µL of RNase A was added to the extract. After completing the extraction, DNA from the two tubes originating from the same sample were pooled. DNA concentrations were measured using a NanoDrop™ spectrophotometer (Thermo Fisher Scientific, Massachusetts, USA).

2.6.2 Amplicon preparation

Fungal communities on the seeds were characterized through amplicon sequencing of the ITS2 region. PCR was performed using the fungal specific primers fITS7 (5'-GTGARTCATCGAATCTTTG-3') (Ihrmark et al. 2012) and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') (White et al. 1990), both of which were extended by a linker base (T), sample-specific 8-base identification tags (which differed in at least three positions), and a terminal base (C) as described in Ihrmark et al. (2012) and Clemmensen et al. (2016).

PCR reactions were carried out in 50-µl volumes containing 0.8 ng/µl template DNA, 200 µM of each dNTP, 2.75 mM MgCl2, 500 nM tagged forward primer, 300 nM tagged reverse primer, and 0.02 U/µl DreamTaq Green polymerase (Thermo Scientific, Massachusetts, USA) in the provided PCR buffer. The thermal cycling conditions included an initial denaturation step at 94°C for 5 minutes, followed by 25–31 cycles of denaturation at 94°C for 30 seconds, annealing at 57°C for 30 seconds, and extension at 72°C for 30 seconds, with a final extension step at 72°C for 7 minutes.

PCR products were purified individually using the AMPure kit (Beckman Coulter, California, USA) and quantified fluorometrically with the Qubit DNA quantification kit (Life Technologies, California, USA). The purified PCR products were pooled and further purified with E.Z.N.A.® Cycle-Pure kit (Omega Bio-Tek, Norcross, Georgia, USA).

The size distribution and quality of all the pools were verified with BioAnalyzer DNA 7500 (Agilent Technologies, California, USA), with ITS2 amplicons ranging between 280 and 380 bp. Concentration and purity were assessed by spectrophotometry (OD 260:280 and 260:230 ratios) using NanoDrop (Thermo Fisher Scientific, Massachusetts, USA). The DNA samples were sequenced at

Science for Life Laboratory (Uppsala University, Sweden) on a Pacific Biosciences Sequel instrument II, using 1 SMRT cell (Pacific Biosciences Inc, California, USA).

2.7 Data analysis

2.7.1 Clustering

SCATA (https://scata.mykopat.slu.se) was used to process, clean and cluster the amplified and sequenced ITS2 fungal reads from the barley kernels (Durling et al. 2011). This was done in accordance with recommendations given by Lindahl et al. (2013). Unprocessed DNA sequences were quality filtered to remove reads shorter than 150 base-pairs. Reads with an error probability greater than 1 in 100 (Score of ≤ 20), and bases with an error probability greater than 1 in 10 (Score of ≤ 5) were discarded. One sample yielded no reads and was discarded. Sequences were screened for primers and tags, sequences with at least a 90% match to the primers, and a 100% match to the tags were preserved. The total number of raw DNA sequences prior to quality control steps totalled 387 734, after filtering and quality control 206 529 reads remained. The following penalties were set for the single-linkage clustering of reads into species-level operational taxonomic units (OTUs) with a clustering distance of 1.5% using SCATA: 1 for base mismatches, 0 for opening a gap in the sequence, 1 for each extension of opened gaps, 0 for gaps at the beginning or end of aligned sequences. The resulting 230 OTUs were preliminarily annotated using RDP classifier (Wang et al. 2007) to identify non-fungal sequences, any identified non-fungal sequence was verified using the NCBI SRA database (https://www.ncbi.nlm.nih.gov/sra). Non-fungal OTUs were mainly of plant origin. The most commonly occurring of these non-fungal OTUs was barley. Finally, OTUs with at least 10 reads across all 100 samples were used for further species annotation and statistical analyses, yielding 83 OTUs in total.

2.7.2 Sequence annotation

Taxonomical annotation was carried out manually on the preliminarily annotated OTUs using PlutoF and UNITE (https://unite.ut.ee/) (Abarenkov et al. 2010; Nilsson et al. 2019). The annotation was made in two stages: The first stage involved assessing the score metric, which measures the mismatches between the query sequence and the fungal sequences in the database, as well as the percentage coverage between the fungal sequence and the query sequence. The second stage focused on examining metadata for the fungal species selected in the first evaluation, including the sequence source, the geographical origin of the sample, interacting taxa, and the total number of samples associated with the specific

species. If the suggested species did not align with the study samples in terms of geographical origin, sequence source, or interacting taxa, the evaluation process was repeated for the second-highest-scoring suggestion.

Three different species complexes were created to accommodate for the limited variability of the ITS2 region in fungi belonging to the *Microdochium* and *Fusarium* genera (O'Donnell et al. 2013). The species complexes were named *Fusarium grp. avenaceum*, *Fusarium grp. poae*, and *Microdochium grp. nivale*. *F. grp. avenaceum* consisted of the species *F. avenaceum*, and *F. tricinctum*. *Fusarium grp. poae* consisted of the species *F. poae, F. oxysporum, F. langsethiae,* and *F. sporotrichioides*. *Microdochium grp. nivale* consisted of the species *M. nivale*, and *M. bolleyi* (O'Donnell et al. 2013; Tsers et al. 2023). Even though many OTUs are annotated at species level, it is possible that this estimation is too generous considering the variability of the ITS2 region and the sequencing depth; closely related species could, for example, benefit from being grouped into species complexes.

After identifying putative species, the read counts for each OTU in each sample were converted into percentages of the total reads across all OTUs in a sample. These percentages were then multiplied by the ergosterol content of each sample, providing a relative estimate of the mass for each OTU present in a sample, expressed as $\frac{mg\ ergosterol}{kg\ cereal}$.

2.7.3 Construction of random forests

The random forest models were constructed using the scikit learn package in Python (Pedregosa et al. 2021). Four classification forests were built to predict gushing, and two regression forests were constructed to predict the percentage of pink kernels. Prior to constructing the models, the data was processed as follows: The gushing measurements were converted from a continuous variable to a true or false binary variable based on two different thresholds: 4 g which is the industry standard and 10 g, respectively. OTU entries belonging to the same species were merged, and the same approach was used to merge OTUs belonging to the same genus. Missing germination data for three samples were imputed by setting them equal to the mean of the germination rates across all samples.

The following parameters were used in the random forest models:

1. *n_estimators*: the number of trees in the forest was set to 500.
2. *max_features*: the number of features to consider when looking for the best split was set to $\sqrt{p}$, as recommended by James et al. 2013.
3. *criterion*: the function used to measure the quality of a split was set to "*gini*" for the Gini impurity index when using gushing as the dependent

variable, and "*squared_error*" for the mean squared error when pink kernels was set as the dependent variable.

4. *random_state*: the seed, which is used by the random number generator was set to 0 prior to any analyses.

The data was split into training and testing datasets using the *train_test_split* function at an 80/20 training-to-testing ratio. The random forest models were fit on the training set and evaluated on the testing set using the F1 score, calculated using the *f1_score* function. Additionally, confusion matrices were constructed for each classification model using the *confusion_matrix* function. To ensure the reliability of the results, the random forest modeling process was repeated 10 times using different random seeds, and the mean and standard deviation of the evaluation metrics (F1 score, out-of-bag error rate, and MSE) were calculated.

2.7.4 NMDS

Non-metric multidimensional scaling (NMDS) analysis was conducted using the vegan package in R (Oksanen et al. 2022). The data used in the NMDS analysis consisted of the OTUs and the geographical region from which each sample originated from, i.e., Köping, Lidköping, or Malmö. The resulting OTU data and metadata were stored in separate dataframes.

The Bray-Curtis dissimilarity matrix was calculated for the species data using the *vegdist* function with the method set to "*bray*". The metaMDS function was then used to perform the NMDS analysis on the dissimilarity matrix with the *trymax* method set to 100 iterations to ensure convergence for the ordination. The resulting NMDS scores were stored in a data frame along with the region information from the metadata. The NMDS scores were then plotted onto scatter plot and coloured by region. In addition, ellipses were drawn around the points of the same region to show the distribution of samples within each region. Ellipses were created for each region using the *stat_ellipse* function with the confidence level set to 0.95 using the method "*level*" which represents 95% of all samples from a given region.

2.7.5 Software and Packages Used for Data Analysis and Visualisations

The creation of histograms utilized Python, employing packages such as pandas (McKinney 2022), along with seaborn (Waskom 2022) and matplotlib (Hunter 2022). Visualisation of hierarchical data was achieved using a krona diagram, generated by a scripted Excel file, accessible at https://github.com/marbl/Krona and credited to Ondov and colleagues in 2011. Bar plots, and the NMDS analysis were generated using the programming language R, and more specifically the

collection of packages named "Tidyverse" and Vegan (Wickham & RStudio 2019; Oksanen et al. 2022).

3. Results

3.1 Sample Characteristics and Distributions

Skewed distributions among gushing and pink kernels | The distribution of pink kernels among the samples was skewed towards the lower values, resulting in an unsymmetrical distribution. The distribution of gushing in g per 330g among the samples was heavily skewed towards the lower values (Fig. 2).

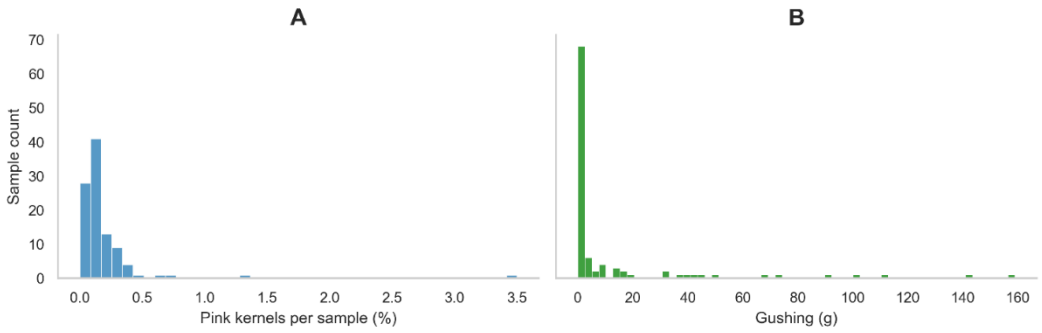

Figure 2. Histograms of barley samples, depicting sample count for (A) the percentage of pink kernels per sample (n=101) and (B) gushing values (gushing g/330g).

3.2 Sample Similarities Across Sites

NMDS reveals no separations between sites | The NMDS analysis conducted on the community data shows no separations between the three sites from which the samples used in this study were gathered from (Fig. 3).

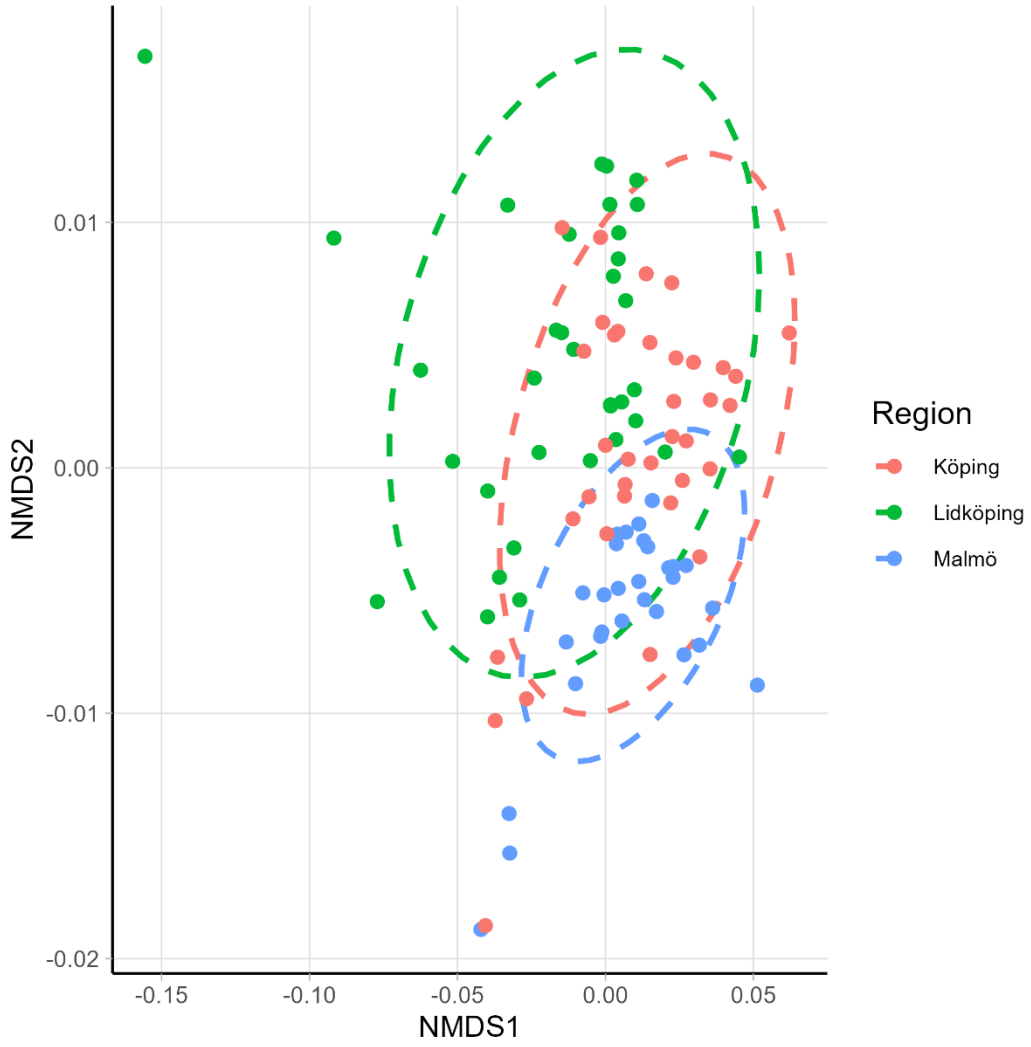

Figure 3. Non-metric multidimensional scaling plot using community data. Each point represents one sample, with colouring of the points based upon regional origin. The dashed ellipses, drawn with a 95% confidence interval, group samples from the same region. Stress value of fit: 0.047.

3.3 Abundance of Fungal Taxa

Four fungal species represent 70% of abundance | The relative abundance and taxonomic distribution of annotated fungal OTUs adjusted for the ergosterol content in each barley sample showed that the 4 most abundant species were *Alternaria infectoria* representing 34% of the community, *Fusarium grp. avenaceum* representing 16% of the community, and *Cladosporium herbarum* and *Vishniacozyma victoriae* (syn. *Cryptococcus victoriae*) both representing 10% of the community each. The most common fungal phylum was Ascomycota. The full list of annotated OTUs is available in table 1, Appendix 1.

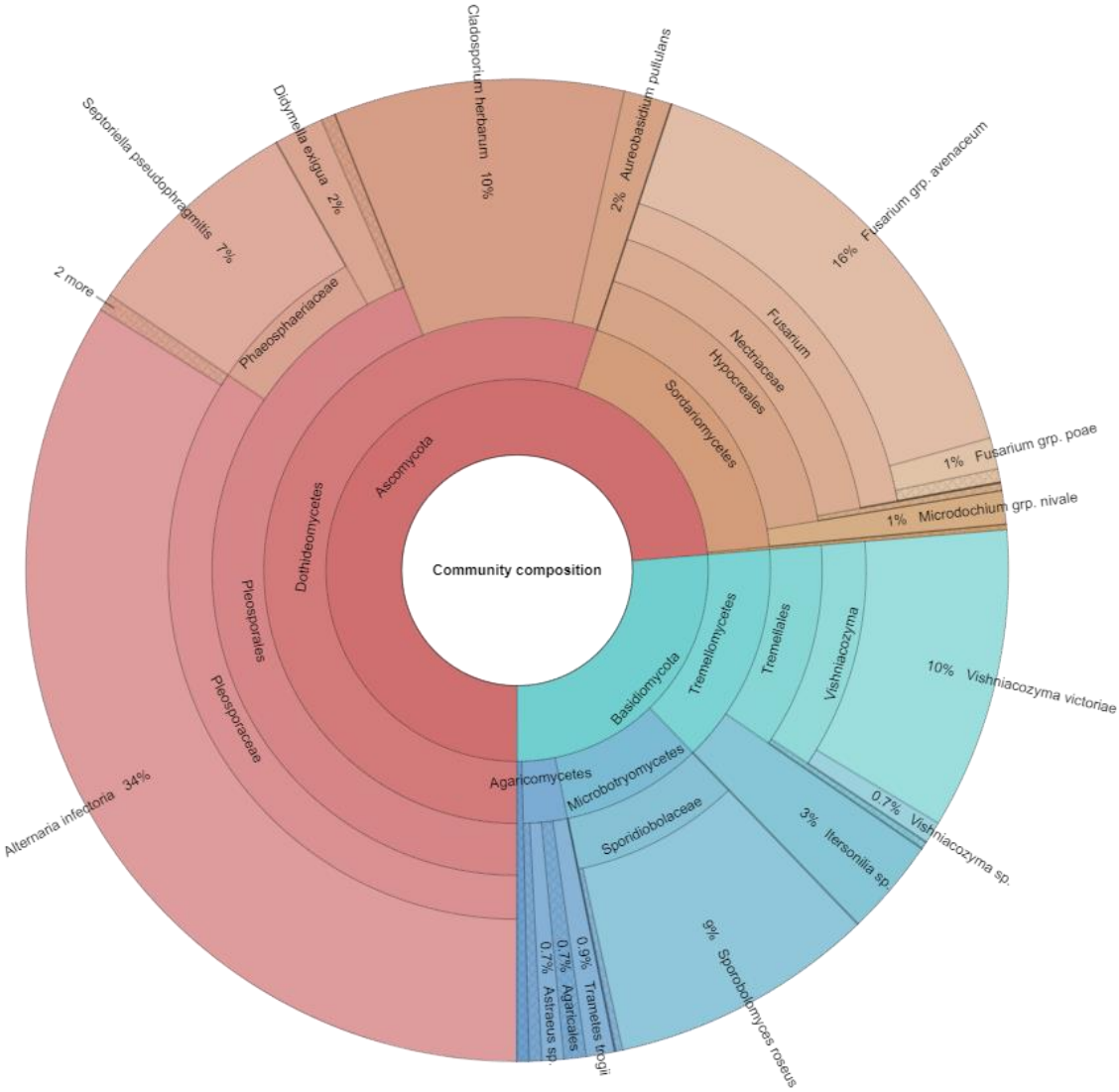

Figure 4. Krona diagram of diversity and relative abundance of annotated fungal OTUs identified in barley kernels. The diagram provides a hierarchical representation of the fungal community, with each level depicting a taxonomic rank (i.e., phylum, class, order, family, genus, and species). The size of each segment indicates the relative abundance of the corresponding taxon. Abundance of OTUs were adjusted for ergosterol content of the samples.

3.4 Fungal OTU Abundance and Community Composition Across Gushing Categories

Increased *Fusarium* abundance in higher gushing-instances | Abundance and community composition of fungal OTUs in barley samples was divided into four distinct gushing categories: 0 g, 1-4 g, 4-10 g, and >10 g. In all gushing categories, *Alternaria infectoria* consistently exhibited the highest mean ergosterol values: 4.43, 4.55, 4.13, and 4.06 mg/kg, respectively. In the highest gushing category *Fusarium grp. avenaceum* demonstrated a four-to-five fold increase in mean ergosterol content (2.65) compared to its values in the other categories (0.58, 0.58, 0.78). Similarly, *Fusarium grp. poae* presented a relatively low mean ergosterol content in the first three categories (0.17, 0.12, 0.17) but experienced a three-fold increase (0.47) in the >10 g gushing category.

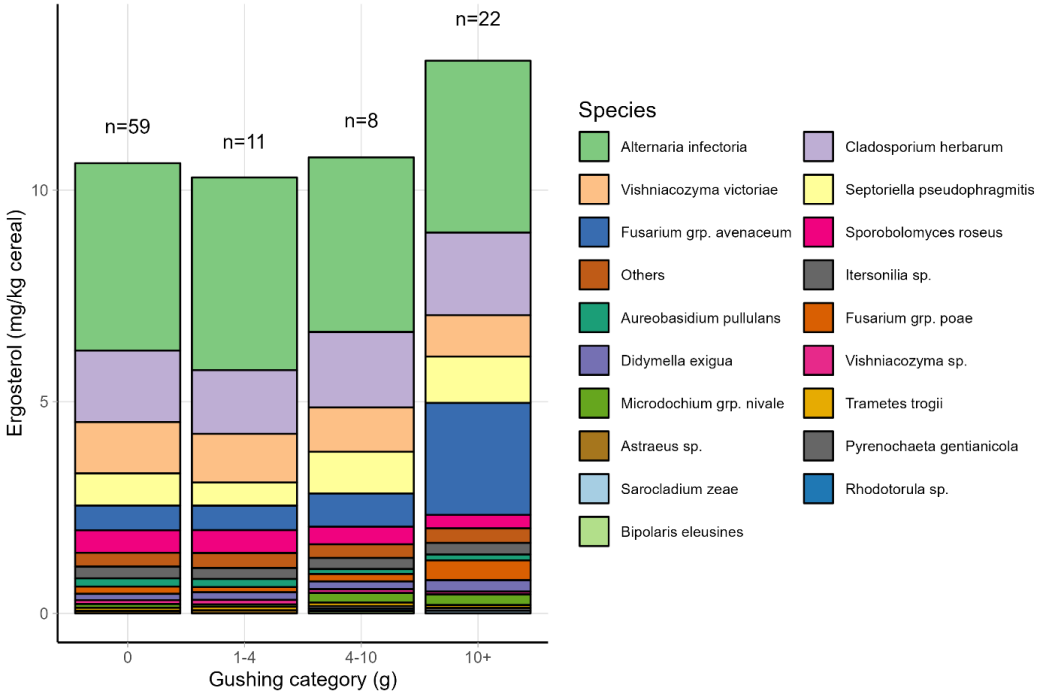

Figure 5. Abundance and community structure of fungal OTUs in barley samples divided by gushing categories (0 g, 1-4 g, 4-10 g, and >10 g). Bar height displays fungal abundance as estimated by ergosterol content of samples in each category. Each bar segment represents a specific fungal OTU. Fungal OTUs with a mean ergosterol value below 0.05 mg/kg cereal were grouped into the category "Others".

3.5 Predicting Gushing: A comparative Analysis of Random Forest Models and Thresholds

***Fusarium* is the most important IV followed by sample independent variables** | Four classification random forest models were developed to assess the relative importance of various IVs in predicting gushing. Two of these models utilized fungal community data at species-level (Fig. 6 & 7), while the other two utilized data at the genus-level (Fig. 8 & 9). In addition to fungal community data, barley sample independent variables including specific weight, total ergosterol content, water content, CgrainA (percentage of pink kernels), protein content, and germination rate were also used as IVs to predict gushing at the two pre-defined thresholds (4g and 10g). The model predicting gushing at a 4 g threshold using data at species-level (Fig. 6) exhibited an out-of-bag (OOB) error rate of 9% and an F1 score of 84%. Similarly, the model predicting gushing at a 10 g threshold (Figure 7) exhibited an OOB error rate of 9% and an F1 score of 84%. None of the models produced any misclassifications, which can be seen in the respective confusion matrices. For the random forest model built using gushing at a 4 g threshold as the dependent variable (Fig. 6), the most important IV identified was *Fusarium grp. avenaceum* (7.7% importance), followed by specific weight (5.5% importance) and total ergosterol content (5.3% importance). Other IVs, including *Fusarium grp. poae* (4.3% importance), *Trametes trogii* (4.2% importance), and *Microdochium grp. nivale* (4.1% importance), contributed to a lesser but comparable degree in determining gushing propensity. For the model using gushing set a 10 g threshold as the dependent variable (Fig. 7), the most influential IV was *Fusarium grp. avenaceum* (7% importance), followed by water content (4.6% importance) and specific weight (4.6% importance). IVs such as total ergosterol (4.4% importance), *Trametes trogii* (4% importance), and *Fusarium grp. poae* (3.9% importance) contributed to a lesser but comparable extent in predicting gushing.

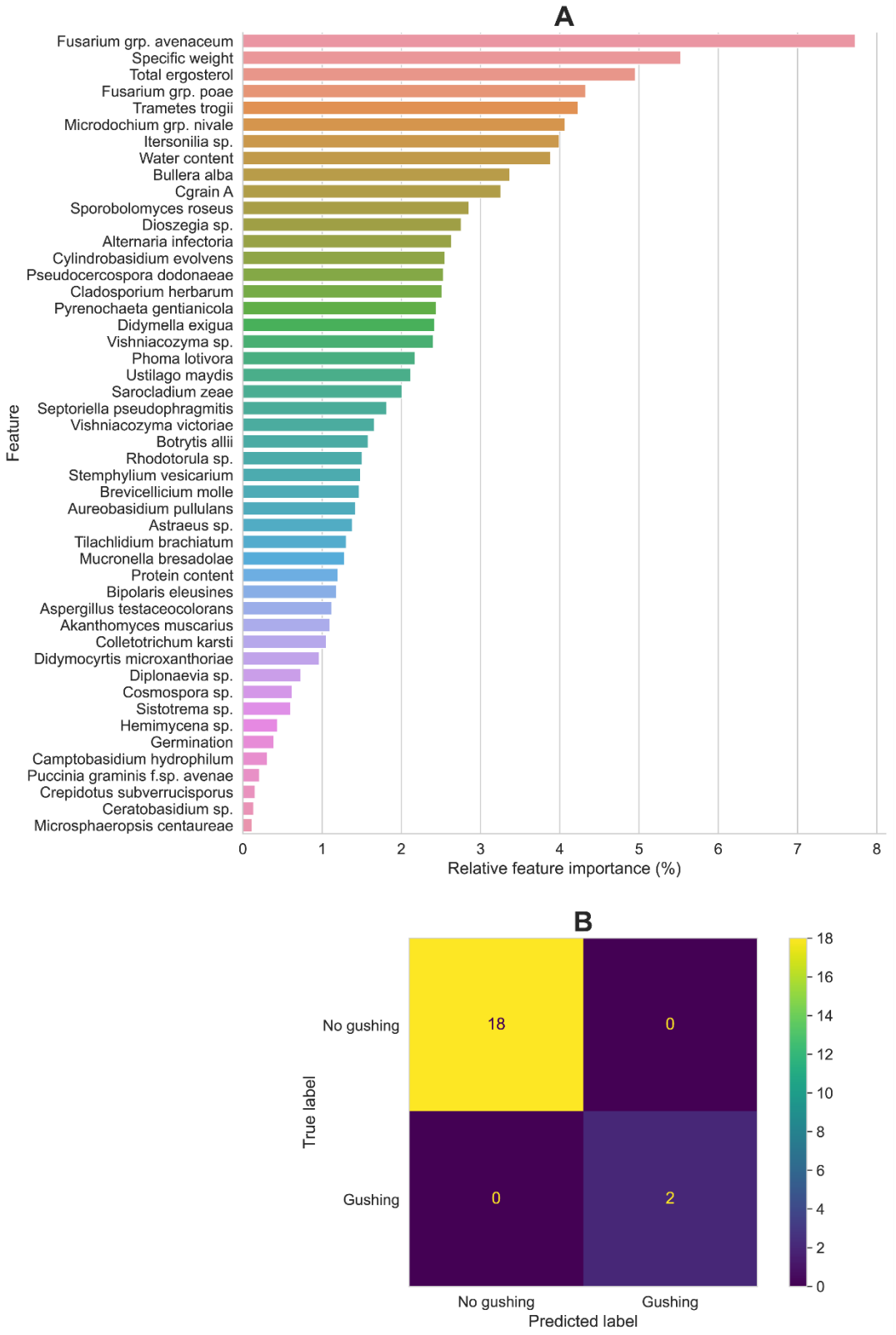

Figure 6. A. Random forest predicting gushing set to a categorical variable using a 4g threshold. Independent variables are the putative fungal species of the annotated OTUs, and related sample independent variables. The model had an F1 score of 0.84±0.1 and an out-of-bag error rate of 0.19±0.02. B. Confusion matrix displaying the classification accuracy for the random forest predicting gushing set to a 4g threshold.

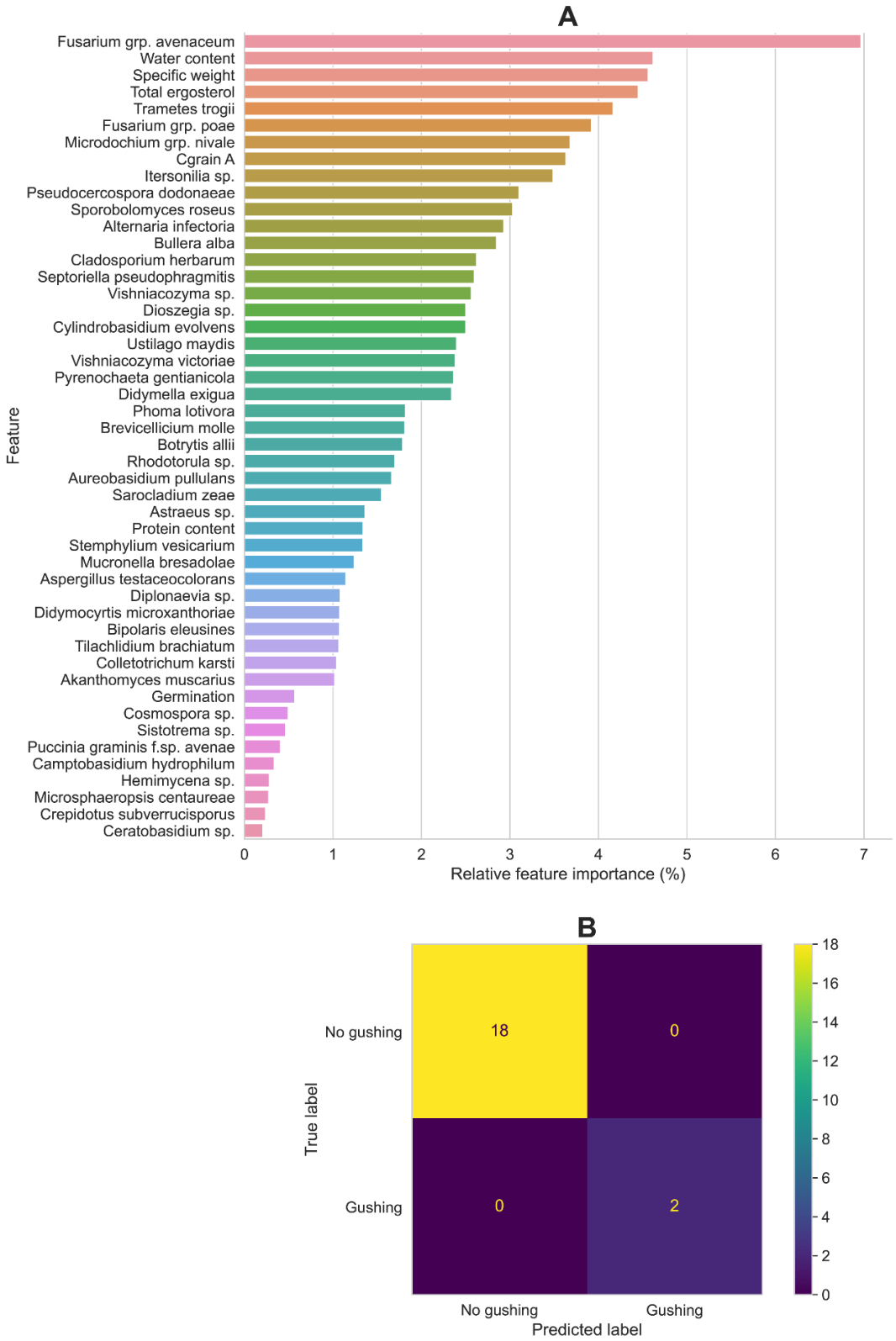

Figure 7. A. Random forest predicting gushing set to a categorical variable using a 10g threshold. Independent variables are the putative fungal species based on the annotated OTUs, and related sample independent variables. The model had an F1 score of 0.84±0.08 and an out-of-bag error rate of 0.19±0.02. B. Confusion matrix displaying the classification accuracy for the random forest predicting gushing set to a 10g threshold.

The genus *Fusarium is* the best predictor of gushing | The model predicting gushing at a 4 g threshold using data at genus level (Figure 8) exhibited an out-of-bag (OOB) error rate of 19% and an F1 score of 82%. Similarly, the model predicting gushing at a 10 g threshold (Figure 9) demonstrated an OOB error rate of 19% and an F1 score of 83%. None of the models produced any misclassifications (Fig 8B & 9B). For the random forest model built using gushing at 4 g threshold as the dependent variable (Figure 8A), the most important IV identified was *Fusarium* (7.9% importance), followed by specific weight (6.1% importance) and water content (5.4% importance). Other IVs, including the basidiomycete *Trametes* (4.6% importance), total ergosterol (4.5% importance), and *Itersonilia* (4.1% importance), contributed to a lesser but comparable degree in determining gushing propensity. For the model using gushing set a 10 g threshold as the dependent variable (Figure 9A), the most influential IV was *Fusarium* (7.8% importance), followed by specific weight (5.5% importance) and *Microdochium* (4.5% importance). IVs such as water content (4.4% importance), total ergosterol (4.4% importance), and *Trametes* (3.9% importance) contributed to a lesser but comparable extent in predicting gushing.

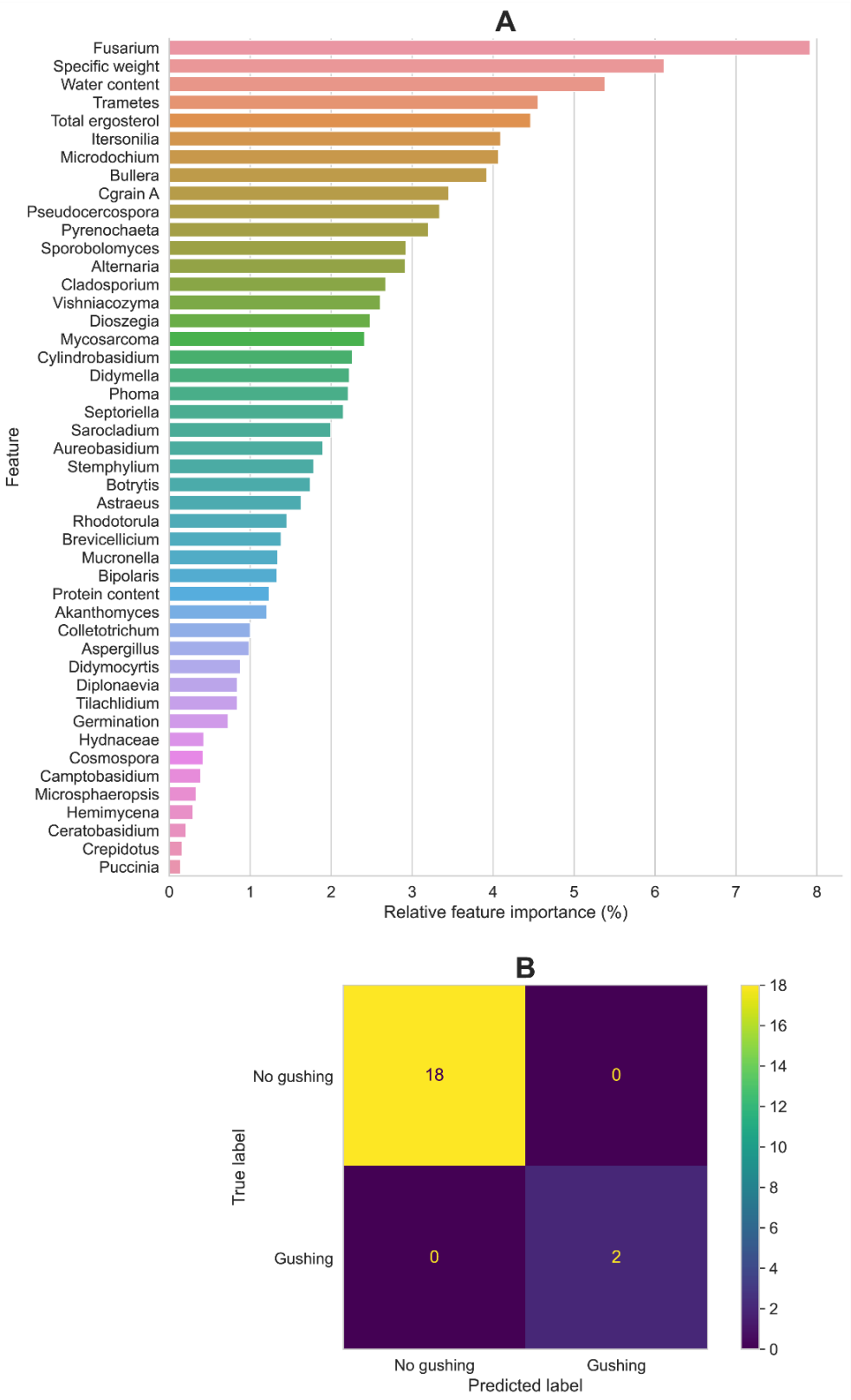

Figure 8. A. Random forest predicting gushing set to a categorical variable using a 4g threshold. Independent variables are fungal genera based on the taxonomy of the annotated OTUs, and related sample independent variables. The model had an F1 score of 0.82±0.09 and an out-of-bag error rate of 0.19±0.03. B. Confusion matrix for random forest predicting gushing set to a 4g threshold.

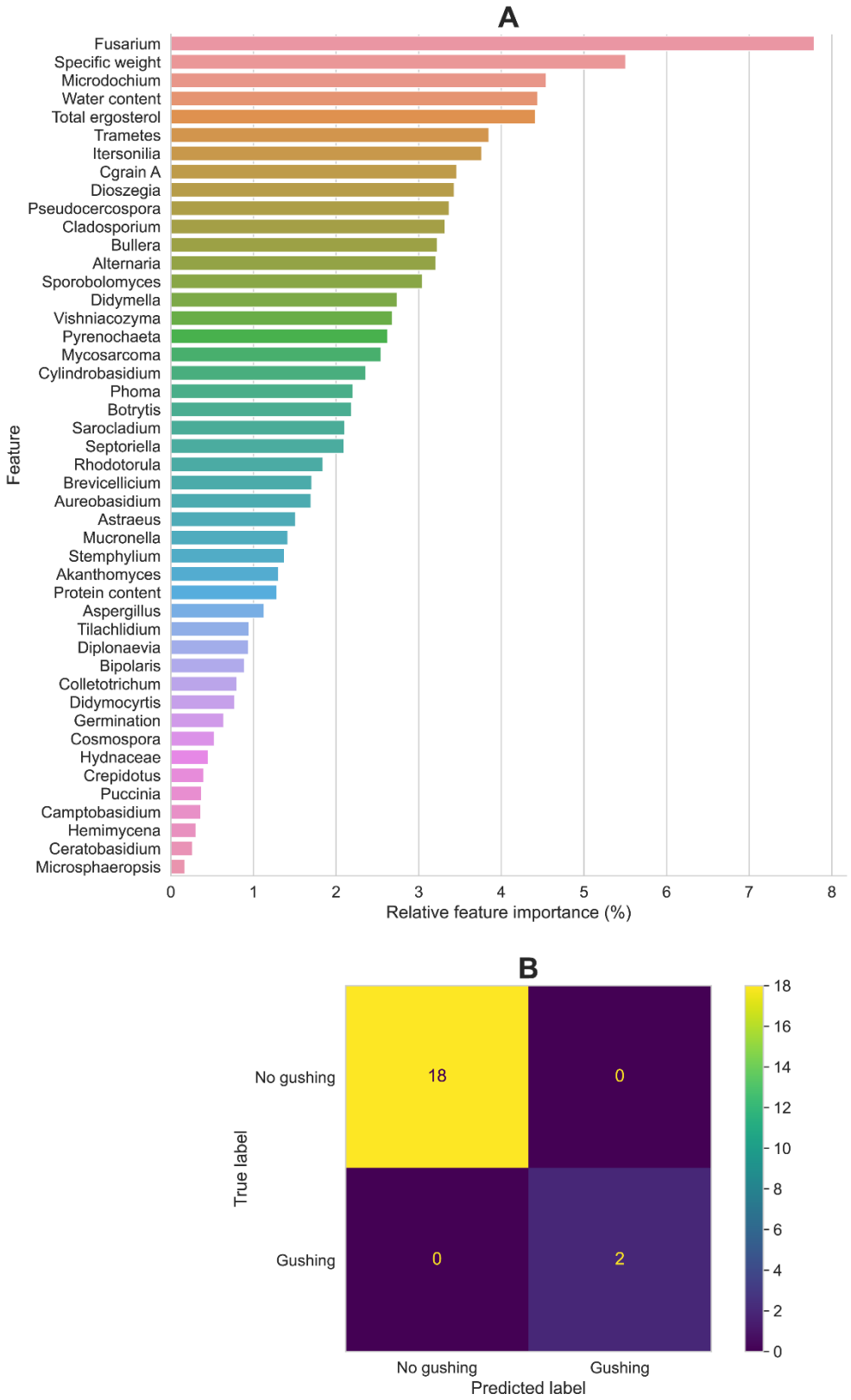

Figure 9. A. Random forest predicting gushing set to a categorical variable using a 10g threshold. Independent variables are fungal genera based on the taxonomy of the annotated OTUs, and related sample independent variables. The model had an F1 score of 0.83±0.08 and an out-of-bag error rate of 0.19±0.03. B. Confusion matrix for random forest predicting gushing set to a 10g threshold.

3.6 Predicting pink kernels: A Comparative Analysis of Species and Genus-level models

***Fusarium* and yeasts are important IVs when predicting pink kernels |** Two regression-based random forest models were developed to assess the relative importance of various IVs in predicting the percentage of pink kernels. Barley sample independent variables (e.g., specific weight, total ergosterol content, water content, CgrainA, protein content, and germination rate) were included as IVs. Moreover, one of these models utilized fungal community data at species-level (Fig. 10), while the other utilized data at the genus-level (Fig. 11). The random forest built to predict the percentage of pink kernels in a sample using data at species-level (Figure 10) identified specific weight as the most important IV (8.4% importance), followed by *Fusarium grp. avenaceum* (8.2% importance), and total ergosterol content (8% importance). The random forest built to predict the percentage of pink kernels in a sample using data at genus-level (Figure 11) identified specific weight as the most important IV (9.2% importance), followed by *Fusarium* (8.2% importance), and *Vishniacozyma* (7.8% importance).

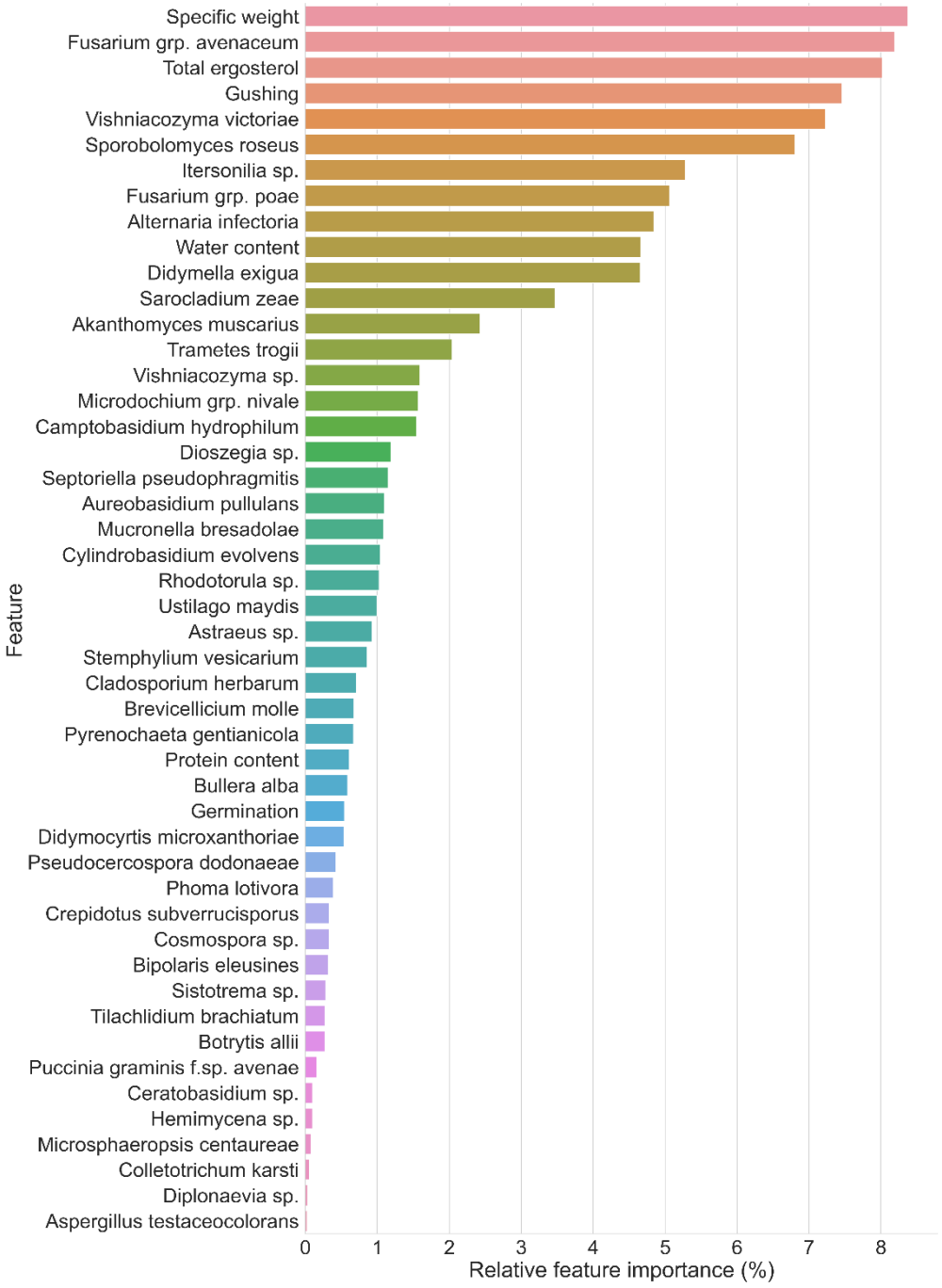

Figure 10. Regression forest predicting the percentage of pink kernels in samples. Independent variables are putative fungal species based on the annotated OTUs, and related sample independent variables. The model had a mean squared error rate of 0.24±0.2 and an out-of-bag error rate of 0.66±0.1.

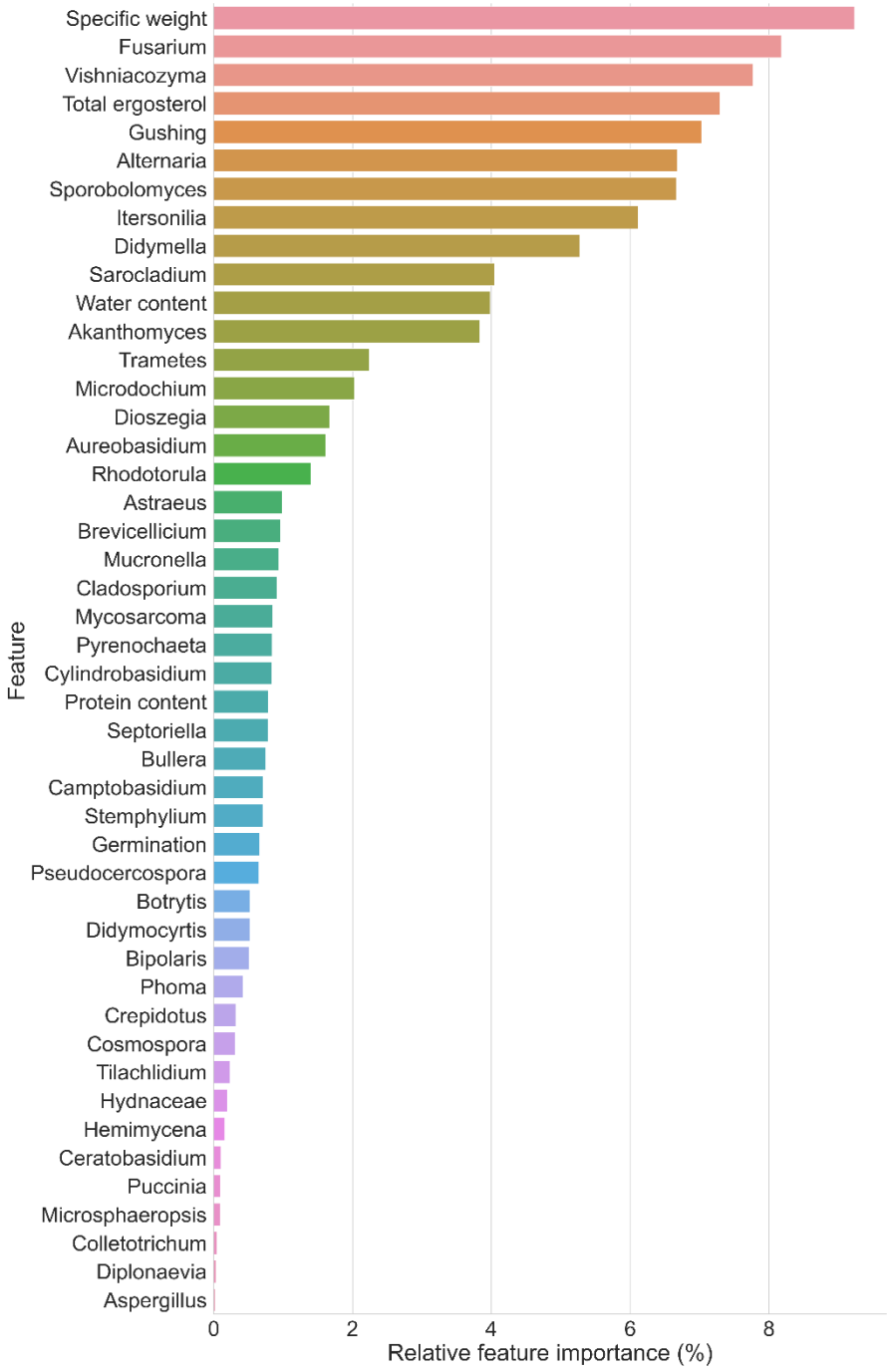

Figure 11. Regression forest predicting the percentage of pink kernels in samples. Independent variables are fungal genera based on the taxonomy of the annotated OTUs, and related sample independent variables. The random forest model had a mean squared error rate of 0.24±0.2 and an out-of-bag error rate of 0.66±0.1.

4. Discussion

4.1 Summary

The present study aimed to investigate the community composition of fungal species on malting barley kernels, and how identified species are associated with gushing-propensity and the presence of pink kernels.

Yeasts with high relative feature importance | When predicting gushing set at two distinct thresholds, using data at both species-, and genus-level the two species complexes of *Fusarium* were identified as the most important IVs. However, many other fungal genera had relatively high feature importance values, when predicting the percentage of pink kernels, yeasts belonging to the fungal genera *Vishniacozyma*, *Itersonilia*, *Dioszegia*, and *Sporobolomyces* were important IVs. *Itersonilia* was also an important fungal genus when predicting gushing set at a 10g threshold but had an even abundance across all gushing categories (Fig. 5). The abundance of *Vishniacozyma*, and *Sporobolomyces* decreased in samples with more gushing (Fig. 5).

The role of *Fusarium* in Swedish malt samples | *Fusarium* being the genus with the highest feature importance across all models is in line with current literature identifying FHB inducing *Fusarium* species as the main gushing-inducing fungal genus, the causality has been shown to be dependent on the production of ascomycete hydrophobins during the malting process. Hydrophobins act as gushing inducing factors during the beer production process (Sarlin et al. 2005a; Virkajärvi et al. 2017b; Geißinger et al. 2022). The results produced in this study further links *Fusarium* to gushing, wherein both *Fusarium* grp. *poae*, and *Fusarium* grp. *avenaceum* increased in the higher gushing categories. However, additional tests for statistical significance are needed between the different categories to validate this hypothesis. Supporting information can be found in current literature on the topic of gushing, indicating *Fusarium* to be highly correlated with gushing, due to the production of hydrophobins (Sarlin et al. 2005a; Geißinger et al. 2022).

4.2 Novel Roles: *Vishniacozyma*, *Microdochium*, and *Itersonilia*

The role of *Vishniacozyma* | The basidiomycete *Vishniacozyma* (incl. the yeast OTUs *V. victoriae* and *V. sp.* identified in this study) is recognized as an environmentally ubiquitous yeast in northern regions (Durán et al. 2019). Vujanovic (2021) reported plant-growth-promoting and mycoparasitic interactions between *V. victoriae* and FHB phytopathogens, corroborating on the finding of *V. victoriae* being the most prevalent basidiomycete as it was present across all gushing categories, but decreased in samples that gushed more (Fig. 5). *V. victoriae* is described to fill a separate ecological niche from filamentous fungi (Vujanovic 2021), which could help explain its consistent presence across all samples in this study. Additionally, the low relative feature importance of *Vishniacozyma* in predicting gushing (2.6%) is supported by the fact that yeasts typically do not produce the hydrophobins that are associated with gushing (Sarlin et al. 2005b). In contrast to the gushing analysis, *V. victoriae* has a higher feature importance when predicting the percentage of pink kernels in a sample (7.8%). Although the cause for this phenomenon remains unclear it is possible that *V. victoriae* causes a similar pinkish discolouration when colonizing the kernel, potentially utilizing similar polyketide pigments as *Fusarium*, which leads to an increase in the estimation of pink kernels, meaning that samples that might not induce gushing could be wrongly classified as containing too many pink kernels even though the discolouration originates from a non-*Fusarium* fungi.(Thomas & Tirumale 2022).

***Microdochium* a similar role as *Fusarium* |** *Microdochium nivale* belongs to the same class as *Fusarium*, namely *Sordariomycetes*, and is recognized as a *Fusarium*-like fungus, sharing many structural similarities (Hernández-Restrepo et al. 2016). However, field infections of *M. nivale* manifest similar symptoms as FHB, the main difference being that *M. nivale* is non-toxigenic, meaning that there is no accumulation of mycotoxins in infected plant material during the infection cycle (Gavrilova et al. 2020). *Microdochium grp. nivale* was identified in this study and constitutes 1% of the fungal community. Despite this, *M. grp. nivale* had a high feature importance when predicting gushing (4.1%), but a relatively low feature importance in predicting pink kernels (2%), indicating an association between *M. grp. nivale* and the gushing phenomenon. This can be attributed to the fact that *M. grp. nivale* produces hydrophobins, as is expected of many filamentous ascomycetes, but lacks the typical pigmentation seen in cultured *Fusarium*. Therefore, samples that could induce gushing might not be detected using pink kernels as indicators if *Microdochium* is abundant, which may affect subsequent production processes such as malting and brewing.

***Itersonilia* another Tremellomycete |** The fungal genus *Itersonilia* harbors known plant pathogens causing petal blight in plants belonging to the *Asteraceae* family, and canker in parsnips and carrots (McGovern et al. 2006). Itersonilia yeasts have been shown to co-occur with *Vishniacozyma* on FHB symptomatic wheat kernels sampled from Denmark and Sweden (Rojas et al. 2020). The relative feature importance of *Itersonilia* mimics the pattern of *Vishniacozyma*, highlighting the possibility that the two fungi share a similar ecological niche.

The role of *Trametes* remains mysterious | *T. trogii* is an environmentally ubiquitous fungi, having been documented on all continents, except for antarctica. *T. trogii* has a saprotrophic lifestyle, primarily as a white-rot fungi decomposing dead wood by employing ligninolytic compounds (Rivera-Hoyos et al. 2013). Hitherto this point, very little literature is available on *T. trogii* being found in agricultural fungal communities, however Gai et al. (2014) found *T. trogii* to be able to degrade wheat straw, highlighting that *T. trogii* can effectively colonize straw tissue, which could possibly explain its presence on stored barley kernels. The low relative abundance of *T. trogii* found in this study together with the high feature importance for predicting gushing (4.2%) and low feature importance (2%) for predicting pink kernels makes *T. trogii* an interesting subject for further studies.

4.3 Sustainable Management of Gushing in Beer Production

By considering a wider array fungal genera besides *Fusarium* and *Microdochium* when evaluating seed lots for gushing propensity, it is possible to improve social, economic, and environmental sustainability aspects tied to the production of malting barley.

4.3.1 Social Dimension: Information Access and Management Strategies

The social dimension | At present, there is a notable deficiency in accessible information for farmers and plant cultivators pertaining to the prevention and management of FHB and the occurrence of pink kernels. Current management suggestions are largely based upon the recommendations devised to mitigate mycotoxin accumulation (Jordbruksverket 2022). Consequently, an assumption has been made that the number of pink kernels in seed lots follow the same pattern as mycotoxin content and that, during years with prevalent mycotoxins, pink kernels must also be widespread (Almén 2020). In terms of species composition, this study has identified fungi that have high feature importance in predicting gushing but not in predicting pink kernels such as *T. trogii*. *Microdochium* is also known to be associated with FHB but is non-toxigenic. This means that using mycotoxin as an indicator during years when *Microdochium* induced FHB is prevalent leads to erroneous estimations of in-field FHB infestations. Such metrics might be misleading and cause malting barley seed lots to be wrongly relegated to animal feed leading to misunderstandings from the farmers perspective, and economic losses. To exemplify the complexity of gushing, sample 80 used in this study had a pink kernel content of 0.3% (i.e., above the industry threshold which is set at 0.1%), ergosterol content of 11 mg/kg cereal and but did not induce any gushing in the MCT analysis. Even so, sample 80 and the corresponding seed lot would have been relegated to animal feed. The findings documented in this study, such as the high feature importance of *Sporobolomyces* could therefore act as a foundation for decision making to be used in developing new management strategies, and in communicating the underlying complexity of gushing and the formation of pink kernels to farmers through agricultural extension practices (Danso-Abbeam et al. 2018).

4.3.2 Economic Dimension: Efficiency, Value Generation, and Food Sovereignty

The economic dimension | The insights generated from the analyses in this study, encompassing malting barley quality variables, gushing propensity, and the identification of novel indicators, not only enhance value generation and increase efficiency in the value chain but also have implications for food sovereignty. By reducing the misclassification of barley seed lots and improving utilization of malting barley, farmers and maltsters can achieve greater control over their production processes and resources. As a result, countries can become more self-reliant in their own food systems, contributing to the broader goal of food sovereignty (Hannah Wittman 2011). This, in turn, supports the rights of farmers to define their own agricultural policies, fostering more diverse production settings and strengthening the resilience of regional food systems in the face of food commodity market disruptions, such as Russia's proposed ban on wheat exports (Glauber et al. 2020). By promoting food sovereignty, these findings contribute to the establishment of sustainable, equitable, and resilient food systems that can better serve the needs of more diverse markets, exemplified by Sweden's beer market.

4.3.3 Environmental Dimension: Pesticide Use and Sustainable Practices

The environmental dimension | The results highlighted in this study also have important implications for the environmental sustainability of malting barley production, particularly in relation to the use of pesticides. At present, the assumption that *Fusarium* is the primary cause of pink discolouration in kernels may lead to unnecessary and excessive application of fungicides targeting this fungal genus (Almén 2020; Jordbruksverket 2022). However, the findings from the present study have revealed that non-*Fusarium* fungal genera may contribute to the formation of pink kernels or gushing propensity, indicating that *Fusarium*-focused fungicide treatments may not always be the most effective or appropriate approach. Indiscriminate use of fungicides can have negative environmental consequences, including harm to non-target organisms, reduction in biodiversity, long-term accumulation in soils, as well as the potential development of pesticide-resistant fungal strains (Yan et al. 2022). Furthermore, the reduction of pesticide use is in-line with goals set by the European Union, as per the Green Deal, therefore unnecessary applications of fungicides should be avoided (Edlinger et al. 2022; Singh et al. 2023). In light of these findings, it is crucial to reconsider current management practices, and to develop strategies that take fungal diversity into account when combating the occurrence of pink kernels and gushing. This may include the use of integrated pest management approaches, which combines

biological, and chemical methods to control pests while minimizing harm done to the environment. Prevention and management of FHB includes the removal of harvest debris and alternative weed hosts that allow *Fusarium* pathogens to persist in fields over longer periods of time, tillage has been shown to be especially effective in cereal-based crop rotations (Suproniene et al. 2019). Adaptation of similar practices, and further research on the topic can aid in discovering more sustainable agroecological solutions which in turn can foster a balance between crop protection, environmental preservation, and long-term agricultural productivity for malting barley producers.

4.4 Random Forest Limitations and Improvements in Study Design

Strengths and weaknesses of RFs | A RF is a machine learning method that can handle smaller datasets so long as the observations are of high quality. This is due to employing bootstrap aggregation, and the inherent strengths of the ensemble characteristic of random forests. To evaluate classification algorithms, confusion matrices, F1 scores, and the OOB error metrics were used, from which it was deduced that the models performed well on the supplied data. However, the performance metrics for the regression random forests were not ideal, with an OOB error rate of 60% and a root mean squared error ($\sqrt{MSE}$) that is greater than the mean. Another noted observation is the variability in the results when using different seeds in the data analysis, this can primarily be attributed to the randomness of the split of the dataset into a training and testing dataset, and secondly to the randomness of the random forest analysis itself, such as the random selection of IVs used to determine the quality of a split when partitioning the data.

Improving study design and analyses | Addressing these limitations can be achieved by increasing the sample size while ensuring less skewed distributions within the samples, improving the accuracy of the annotations, and assessing alternative machine learning techniques or performance metrics, such as permutation random forests, and gradient boosting classifiers. Additionally, incorporating significance testing may provide a more robust understanding of the relationships between the composition of fungal communities, gushing propensity, and the presence of pink kernels.

4.5 Conclusions and Future Research

4.5.1 Conclusions

This study showed that (I) A plethora of different fungal species, including yeasts and filamentous fungi, compose the communities in barley grains, species belonging to the fungal genera *Fusarium*, *Microdochium*, *Vishniacozyma*, *Alternaria*, and *Cladosporium* were present across samples analysed in this study. (II) Among other species identified in this study, *F. grp. poae*, *F. grp. avenaceum*, T. *trogii*, and *M. grp. nivale* were identified as the most important OTUs for predicting gushing. (III) Additionally, *F. grp. avenaceum*, *V. victoriae*, *S. roseus*, *Itersonilia sp.*, and *F. grp. poae* are the OTUs with the highest relative feature importance for predicting the percentage of pink kernels in a sample. It is essential to mention that this study was conducted using samples from only one growing season and across three sites in Southern Sweden, as such, broader implications are only discussed. Understanding how the identified species induce gushing or the formation of pink kernels will require further research.

4.5.2 Future research

Metagenomics – who is there? | Building on the findings of this study, there are several promising avenues for future research that can provide a greater context for gushing-, and barley-related research. The current study has provided insights into the fungal species present in barley grains, and their impact on gushing, as well as the presence of pink kernels, answering the question "Who is there?" but not "What are they doing?". Further research is therefore needed to establish a direct causality between the species identified in this study, and their respective roles in inducing gushing, and their ability to form pink kernels.

Metabolomics – what are they doing? | In this study amplicon sequencing and metagenomics has been utilized to disseminate the species present in fungal communities in barley grains to answer the question *"Who is there?"*. To discuss the role or function of species of interest, relevant literature has been contextualized and analysed. The next step could be to verify this discussion by employing metabolomics, which is defined as a comprehensive and quantitative analysis of all metabolites in a biological system (Fiehn 2001). New insights can be gained by trying to understand how hitherto overlooked fungal species contribute to gushing by screening for secondary metabolites as constituents of their secretome or metabolome. Indeed, this has already been utilized to assess mycotoxin production and accumulation caused by the *Fusarium* species complex (Lowe et al. 2010). A similar analysis can be done to unveil the metabolome of *T. trogii*, and other relevant species identified in this study to verify their ability to induce gushing or to induce the formation of pink kernels.

Greenhouse experiments – artificial inoculation | Understanding fungal community interactions over time allows researchers to assess and characterize possible synergistic, or antagonistic interactions between species. To exemplify this, an *in-situ* study by Rojas et al. (2020) assessed the spatio-temporal shifts of fungal genera in communities on wheat spikes. Their findings indicate that there is both niche exclusion by *Fusarium*, and a negative correlation between *Fusarium* and other endophytes. Similar *Ex-situ* greenhouse experiments with, for example, artificial inoculation of pairs of species would allow for researchers to disseminate beneficial or antagonistic interactions between species, which could potentially aid in developing methodologies that can aid in determining the causality between the formation of pink kernels and gushing.

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Popular scientific summary: Fungal Intruders: The Unseen Threat to Your Beer

Are you a beer enthusiast, or perhaps, part of the beer production industry? Do you find that high quality beer is important to you? Then this read might be worth your while!

Fungal contamination in malting barley could secretly be causing problems in your beer - from changing the colour of barley to inducing a fizzy gushing of beer upon opening. Our investigation delved into these minute, unseen invaders of barley grains – the foundation for malt beer, unearthing new culprits and underlining their impact on beer production. In the humble fields of southern Sweden, a silent drama unfolds. Here, fungi invisible to the naked eye, are waging a war on the malting barley we all know and love. And their actions are causing what we refer to as *"gushing"* - that unexpected overflow of foam and beer you might have experienced when popping the cap of a beer bottle.

To put this into context, think of these fungi as uninvited guests at a party, causing quite the commotion. In this case, the party is the barley grains, and the commotion is gushing, the afterparty is the production of vigorous amounts of foam when beer containers are de-pressurized or opened. Understanding these party crashers and their antics was the goal of our study, which scoured through barley samples from southern Sweden, investigating the influence of these fungi on the raw materials of beer. Our aim was to characterise the fungal communities dwelling within the sampled barley grains, and to examine to which extent these fungal communities contribute to gushing, and the pinkish discolouration of barley grains – which can be seen as an indication of grain quality.

We noted that among the identified fungal species a well-known fungal plant-pathogen named *Fusarium* contributed the most to both the discolouration of grain and to gushing in our samples – measured via model importance. Surprisingly however, other fungal genera were found to discolour the barley grains in a similar manner as *Fusarium*, including two types of yeast, which have previously garnered little attention in the context of grain quality research; and like unmasking new suspects, their unforeseen roles had gone unseen until now. To conclude the results

from this project there seems to be many different fungi contributing to the gushing phenomenon at different stages of the malt beer production process.

So, what does this mean for the beer we all know and love? The identification of these fungal culprits offers a step forward in improving the quality of beer. It equips the industry with new knowledge which can be used to develop targeted strategies, improve quality assessments, and reduce economic losses due to the contamination of malting barley grains. These microscopic invaders can significantly influence the fate of our favourite beverage. Monitoring and managing these party crashers could mean more sustainable beer production and, ultimately, a perfect pint in your hand. However, just like with beer foam, what you see is just the froth. More research is needed to unravel the complete role of these fungi and to find effective ways to control them. The battle against these invisible invaders continues, all in pursuit of the perfected brew. One thing remains clear, in this intricate world of fungi and barley, even the smallest of organisms can have the most profound of impacts on your after-work beer.

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Appendix 1

Table 1. List of all annotated clusters and OTUs used in statistical analyses.

Cluster name	Read counts	Phylum	Class	Order	Family	Genus	Species
scata5511_0	15960	Ascomycota	Dothideomycetes	Pleosporales	Pleosporaceae	*Alternaria*	*Alternaria infectoria*
scata5511_1	4409	Ascomycota	Sordariomycetes	Hypocreales	Nectriaceae	*Fusarium*	*Fusarium grp. avenaceum*
scata5511_2	13242	Ascomycota	Dothideomycetes	Capnodiales	Cladosporiaceae	*Cladosporium*	*Cladosporium herbarum*
scata5511_3	4835	Basidiomycota	Microbotryomycetes	Sporidiobolales	Sporidiobolaceae	*Sporobolomyces*	*Sporobolomyces roseus*
scata5511_4	3286	Basidiomycota	Tremellomycetes	Tremellales	Bulleribasidiaceae	*Vishniacozyma*	*Vishniacozyma victoriae*
scata5511_5	4579	Ascomycota	Dothideomycetes	Pleosporales	Phaeosphaeriaceae	*Septoriella*	*Septoriella pseudophragmitis*
scata5511_6	5974	Ascomycota	Dothideomycetes	Pleosporales	Pleosporaceae	*Alternaria*	*Alternaria infectoria*
scata5511_8	1672	Ascomycota	Dothideomycetes	Dothideales	Saccotheciaceae	*Aureobasidium*	*Aureobasidium pullulans*
scata5511_9	1344	Ascomycota	Dothideomycetes	Pleosporales	Didymellaceae	*Didymella*	*Didymella exigua*
scata5511_10	749	Basidiomycota	Tremellomycetes	Cystofilobasidiales	Cystofilobasidiaceae	*Itersonilia*	*Itersonilia sp.*
scata5511_11	1683	Ascomycota	Dothideomycetes	Pleosporales	Pleosporaceae	*Alternaria*	*Alternaria infectoria*
scata5511_12	864	Basidiomycota	Tremellomycetes	Cystofilobasidiales	Cystofilobasidiaceae	*Itersonilia*	*Itersonilia sp.*
scata5511_13	780	Basidiomycota	Agaricomycetes	Polyporales	Polyporaceae	*Trametes*	*Trametes trogii*
scata5511_14	720	Ascomycota	Dothideomycetes	Pleosporales	Phaeosphaeriaceae	*Septoriella*	*Septoriella pseudophragmitis*
scata5511_15	633	Basidiomycota	Agaricomycetes	Boletales	Diplocystaceae	*Astraeus*	*Astraeus sp.*
scata5511_16	513	Basidiomycota	Tremellomycetes	Tremellales	Bulleribasidiaceae	*Vishniacozyma*	*Vishniacozyma sp.*
scata5511_17	903	Ascomycota	Sordariomycetes	Hypocreales	Nectriaceae	*Fusarium*	*Fusarium grp. poae*
scata5511_18	812	Basidiomycota	Tremellomycetes	Tremellales	Bulleribasidiaceae	*Vishniacozyma*	*Vishniacozyma victoriae*
scata5511_19	680	Ascomycota	Sordariomycetes	Xylariales	Microdochiaceae	*Microdochium*	*Microdochium grp. nivale*
scata5511_20	433	Basidiomycota	Tremellomycetes	Cystofilobasidiales	Cystofilobasidiaceae	*Itersonilia*	*Itersonilia sp.*
scata5511_21	434	Ascomycota	Dothideomycetes	Pleosporales	Cucurbitariaceae	*Pyrenochaeta*	*Pyrenochaeta gentianicola*
scata5511_22	305	Basidiomycota	Agaricomycetes	Trechisporales	Hydnodontaceae	*Brevicellicium*	*Brevicellicium molle*
scata5511_23	297	Ascomycota	Sordariomycetes	Hypocreales	Nectriaceae	*Fusarium*	*Fusarium grp. poae*
scata5511_24	227	Basidiomycota	Agaricomycetes	Agaricales	Clavariaceae	*Mucronella*	*Mucronella bresadolae*
scata5511_25	346	Ascomycota	Dothideomycetes	Pleosporales	Pleosporaceae	*Bipolaris*	*Bipolaris eleusines*
scata5511_26	315	Ascomycota	Sordariomycetes	Hypocreales	Sarocladiaceae	*Sarocladium*	*Sarocladium zeae*
scata5511_27	350	Ascomycota	Sordariomycetes	Hypocreales	Nectriaceae	*Fusarium*	*Fusarium grp. poae*

Cluster name	Read counts	Phylum	Class	Order	Family	Genus	Species
scata5511_28	313	Ascomycota	Sordariomycetes	Xylariales	Microdochiaceae	*Microdochium*	*Microdochium grp. nivale*
scata5511_29	293	Ascomycota	Dothideomycetes	Pleosporales	Phaeosphaeriaceae	*Septoriella*	*Septoriella pseudophragmitis*
scata5511_30	253	Basidiomycota	Agaricomycetes	Agaricales	Physalacriaceae	*Cylindrobasidium*	*Cylindrobasidium evolvens*
scata5511_31	225	Basidiomycota	Tremellomycetes	Tremellales	Tremellaceae	*Dioszegia*	*Dioszegia sp.*
scata5511_32	214	Basidiomycota	Microbotryomycetes	Sporidiobolales	Sporidiobolaceae	*Rhodotorula*	*Rhodotorula sp.*
scata5511_33	3	Ascomycota	Sordariomycetes	Xylariales	Microdochiaceae	*Microdochium*	*Microdochium grp. nivale*
scata5511_34	64	Ascomycota	Dothideomycetes	Pleosporales	Phaeosphaeriaceae	*Septoriella*	*Septoriella pseudophragmitis*
scata5511_35	138	Ascomycota	Dothideomycetes	Pleosporales	Pleosporaceae	*Stemphylium*	*Stemphylium vesicarium*
scata5511_36	189	Basidiomycota	Ustilaginomycetes	Ustilaginales	Ustilaginaceae	*Mycosarcoma*	*Ustilago maydis*
scata5511_37	189	Ascomycota	Leotiomycetes	Helotiales	Sclerotiniaceae	*Botrytis*	*Botrytis allii*
scata5511_38	244	Ascomycota	Sordariomycetes	Hypocreales	Nectriaceae	*Fusarium*	*Fusarium grp. poae*
scata5511_39	133	Basidiomycota	Tremellomycetes	Cystofilobasidiales	Cystofilobasidiaceae	*Itersonilia*	*Itersonilia sp.*
scata5511_40	151	Basidiomycota	Tremellomycetes	Cystofilobasidiales	Cystofilobasidiaceae	*Itersonilia*	*Itersonilia sp.*
scata5511_41	123	Basidiomycota	Microbotryomycetes	Sporidiobolales	Sporidiobolaceae	*Sporobolomyces*	*Sporobolomyces roseus*
scata5511_42	88	Basidiomycota	Tremellomycetes	Cystofilobasidiales	Cystofilobasidiaceae	*Itersonilia*	*Itersonilia sp.*
scata5511_43	84	Basidiomycota	Ustilaginomycetes	Ustilaginales	Ustilaginaceae	*Mycosarcoma*	*Ustilago maydis*
scata5511_45	56	Basidiomycota	Agaricomycotina	Tremellales	Bulleraceae	*Bullera*	*Bullera alba*
scata5511_46	46	Basidiomycota	Tremellomycetes	Cystofilobasidiales	Cystofilobasidiaceae	*Itersonilia*	*Itersonilia sp.*
scata5511_47	4	Ascomycota	Dothideomycetes	Pleosporales	Pleosporaceae	*Alternaria*	*Alternaria infectoria*
scata5511_49	15	Ascomycota	Dothideomycetes	Pleosporales	Phaeosphaeriaceae	*Septoriella*	*Septoriella pseudophragmitis*
scata5511_50	116	Ascomycota	Dothideomycetes	Pleosporales	Phoma	*Phoma*	*Phoma lotivora*
scata5511_51	31	Basidiomycota	Tremellomycetes	Tremellales	Bulleribasidiaceae	*Vishniacozyma*	*Vishniacozyma sp.*

Cluster name	Read counts	Phylum	Class	Order	Family	Genus	Species
scata5511_53	18	Basidiomycota	Tremellomycetes	Tremellales	Tremellaceae	*Dioszegia*	*Dioszegia sp.*
scata5511_54	21	Basidiomycota	Microbotryomycetes	Kriegeriales	Camptobasidiaceae	*Camptobasidium*	*Camptobasidium hydrophilum*
scata5511_55	27	Ascomycota	Dothideomycetes	Pleosporales	Phaeosphaeriaceae	*Septoriella*	*Septoriella pseudophragmitis*
scata5511_57	30	Ascomycota	Dothideomycetes	Pleosporales	Phaeosphaeriaceae	*Septoriella*	*Septoriella pseudophragmitis*
scata5511_58	72	Ascomycota	Sordariomycetes	Hypocreales	Nectriaceae	*Fusarium*	*Fusarium grp. poae*
scata5511_59	8	Basidiomycota	Agaricomycotina	Cantharellales	Ceratobasidiaceae	*Ceratobasidium*	*Ceratobasidium sp.*
scata5511_60	69	Basidiomycota	Ustilaginomycetes	Ustilaginales	Ustilaginaceae	*Mycosarcoma*	*Ustilago maydis*
scata5511_63	67	Ascomycota	Sordariomycetes	Hypocreales	Tilachlidiaceae	*Tilachlidium*	*Tilachlidium brachiatum*
scata5511_65	5	Ascomycota	Dothideomycetes	Pleosporales	Phaeosphaeriaceae	*Septoriella*	*Septoriella pseudophragmitis*
scata5511_66	244	Ascomycota	Sordariomycetes	Xylariales	Microdochiaceae	*Microdochium*	*Microdochium grp. nivale*
scata5511_69	35	Ascomycota	Sordariomycetes	Hypocreales	Nectriaceae	*Cosmospora*	*Cosmospora sp.*
scata5511_72	45	Basidiomycota	Tremellomycetes	Tremellales	Bulleraceae	*Bullera*	*Bullera alba*
scata5511_77	29	Ascomycota	Eurotiomycetes	Eurotiales	Aspergillaceae	*Aspergillus*	*Aspergillus testaceocolorans*
scata5511_81	30	Ascomycota	Sordariomycetes	Xylariales	Microdochiaceae	*Microdochium*	*Microdochium grp. nivale*
scata5511_83	9	Ascomycota	Dothideomycetes	Pleosporales	Phaeosphaeriaceae	*Septoriella*	*Septoriella pseudophragmitis*
scata5511_85	59	Basidiomycota	Ustilaginomycetes	Ustilaginales	Ustilaginaceae	*Mycosarcoma*	*Ustilago maydis*
scata5511_88	17	Basidiomycota	Tremellomycetes	Tremellales	Bulleribasidiaceae	*Vishniacozyma*	*Vishniacozyma sp.*
scata5511_92	15	Basidiomycota	Agaricomycetes	Agaricales	Mycenaceae	*Hemimycena*	*Hemimycena sp.*
scata5511_93	11	Ascomycota	Dothideomycetes	Pleosporales	Microsphaeropsidaceae	*Microsphaeropsis*	*Microsphaeropsis centaureae*
scata5511_95	157	Ascomycota	Sordariomycetes	Hypocreales	Cordycipitaceae	*Akanthomyces*	*Akanthomyces muscarius*
scata5511_108	20	Ascomycota	Dothideomycetes	Dothideales	Saccotheciaceae	*Aureobasidium*	*Aureobasidium pullulans*
scata5511_109	29	Ascomycota	Sordariomycetes	Glomerellales	Glomerellaceae	*Colletotrichum*	*Colletotrichum karsti*

Cluster name	Read counts	Phylum	Class	Order	Family	Genus	Species
scata5511_124	15	Basidiomycota	Tremellomycetes	Cystofilobasidiales	Cystofilobasidiaceae	*Itersonilia*	*Itersonilia sp.*
scata5511_130	15	Ascomycota	Sordariomycetes	Hypocreales	Nectriaceae	*Cosmospora*	*Cosmospora sp.*
scata5511_142	29	Ascomycota	Dothideomycetes	Pleosporales	Pleosporaceae	*Alternaria*	*Alternaria infectoria*
scata5511_154	26	Ascomycota	Dothideomycetes	Pleosporales	Phaeosphaeriaceae	*Didymocyrtis*	*Didymocyrtis microxanthoriae*
scata5511_167	11	Basidiomycota	Ustilaginomycetes	Ustilaginales	Ustilaginaceae	*Mycosarcoma*	*Ustilago maydis*
scata5511_168	16	Basidiomycota	Microbotryomycetes	Sporidiobolales	Sporidiobolaceae	*Rhodotorula*	*Rhodotorula sp.*
scata5511_179	36	Basidiomycota	Agaricomycetes	Agaricales	Crepidotaceae	*Crepidotus*	*Crepidotus subverrucisporus*
scata5511_181	19	Basidiomycota	Pucciniomycetes	Pucciniales	Pucciniaceae	*Puccinia*	*Puccinia graminis f.sp. avenae*
scata5511_196	14	Basidiomycota	Agaricomycetes	Cantharellales	Cystofilobasidiaceae	*Hydnaceae*	*Sistotrema sp.*
scata5511_199	16	Ascomycota	Leotiomycetes	Helotiales	Calloriaceae	*Diplonaevia*	*Diplonaevia sp.*
scata5511_208	24	Ascomycota	Dothideomycetes	Mycosphaerellales	Mycosphaerellaceae	*Pseudocercospora*	*Pseudocercospora dodonaeae*

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